ASSESSING CONSERVED FUNCTION OF CONIDIATION REGULATORS IN TWO DISTANTLY RELATED ASCOMYCETES, *Aspergillus nidulans* AND *Neurospora crassa*

A Dissertation

by

DA WOON CHUNG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Brian D. Shaw
Committee Members,	Daniel J. Ebbole
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ABSTRACT

Assessing Conserved Function of Conidiation Regulators in Two Distantly Related Ascomycetes, *Aspergillus nidulans* and *Neurospora crassa*. (May 2011)

Da Woon Chung, B.S., Ewha Womans University, Seoul, South Korea; M.S., Seoul National University, Seoul, South Korea; M.S., University of Wisconsin-Madison

Chair of Advisory Committee: Dr. Brian D. Shaw

Conidiation is a common and critical asexual reproductive mode in fungi. The ascomycetes, the largest group in the kingdom Fungi undergo conidiation. The wide array of morphological differences in conidiophores and conidial size, shape, and cellular organization demonstrates the importance of evolution in driving the morphological and functional diversity. An important unanswered question is how these conidiation processes evolve. We hypothesized that a conidiation regulatory pathway was present in the ancestral species, and became specialized in the extant species to lead to morphological and functional diversity. To address this hypothesis we assessed the conserved function of conidiation regulators in two distantly related ascomycetes, *Aspergillus nidulans* and *Neurospora crassa*. Using sequence similarity analysis, *N. crassa* orthologs were characterized to seven main conidiation regulatory genes in *A. nidulans* (*fluG*, *flbC*, *flbD*, *abaA*, *wetA*, *medA*, and *stuA*). Expression of the *N. crassa* orthologs complemented defective conidiation in the *A. nidulans fluG*, *flbD*, *wetA*, *medA*, and *stuA* mutants. In contrast, *abaA* and *flbC* and the *N. crassa* orthologs did not

share conserved biochemical function. Taken in context of other recent studies of conidiation regulators, there are four distinct evolutionary patterns: (i) Non-homologous genes with analogous roles in conidiation ('*brlA*' and '*fl*'), (ii) Orthologs with retained biochemical function that lack analogous role in conidiation ('*fluG*', '*flbD*', and '*wetA*'), (iii) Orthologs with retained biochemical function and analogous roles in conidiation ('*medA*' and '*stuA*'), and (iv) Orthologs with biochemical function not conserved but with analogous roles in conidiation ('*abaA*' and '*flbC*'). These studies set the stage for long-term studies of how evolution proceeded during the evolution of conidiation at different levels of phylogenetic diversity. An understanding of how evolutionary mechanisms shape the dynamics of developmental pathways will be significant for our understanding of fungal evolution of other novel adaptations such as pathogenesis.

DEDICATION

To my parents, Wan Gyun Chung and Sook Gyong Lim, and to my sisters, Yoo Jin Chung and Ye Jin Chung for their love and support.

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CHAPTER I

INTRODUCTION

CONIDIATION IN FUNGI

Conidiation is an asexual reproductive mode in a diverse group of fungi including important species that impact agriculture, industry, and medicine (Adams et al., 1998). Conidia are propagules for dissemination, inocula for fungal infection to plants and animals, and can survive in extreme environments (Adams et al., 1998; Ebbole, 2010). Fungal species in the phylum Ascomycota (generally called 'ascomycetes') constitute the largest phylum in the fungal kingdom. It has been reported that more than 15,000 described ascomycetes species reproduce only asexually (Cole, 1986). Due to its commonality and importance in fungal biology, conidiation has been studied in a diverse group of fungi. In addition, conidiation regulatory genes and the genetic pathways also have been characterized in *Penicillium* spp., *N. crassa*, and A. *nidulans* (Roncal and Ugalde, 2003; Springer and Yanofsky, 1989; Timberlake and Marshall, 1988). However, how the conidiation regulators have evolved across the fungal taxa to coordinate conidiation remains poorly understood.

Conidia, the asexual spores, are produced from a multicellular spore-bearing structure called a conidiophore. Conidiophores form in a colony of vegetative hyphae induced by certain environmental stimuli including aerial cues (desiccation and hyperoxia), light, osmotic stress, and nutrition limitation (Roncal and Ugalde, 2003). For

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This dissertation follows the style of Fungal Genetics and Biology.

both *A. nidulans* and *N. crassa*, conidiophore formation does not occur for wild type hyphae grown in submerged culture, which suggests that the exposure of hyphae to the air is critical to induction of conidiation in *A. nidulans* (Adams et al., 1998; Ebbole, 2010). In *A. nidulans*, high osmolarity such as 0.8 M NaCl induces conidiation in submerged culture that *A. nidulans* wild type normally did not prefer to conidiate (Lee and Adams, 1996). In *N. crassa*, light is a positive stimulus for conidiation, and White Collar-1 (WC-1) proteins are characterized as blue light photoreceptors that regulate light induction of circadian clock (He et al., 2002). In addition, limitation of carbon sources can induce conidiation of *N. crassa* wild type in submerged culture (Madi et al., 1997).

CONIDIATION OF *Aspergillus nidulans*

Conidiophore formation in *A. nidulans* initiates from a specialized foot cell with a thickened cell wall differentiated from vegetative hyphae. The foot cell gives rise to an aerial stalk (a diameter 4 – 5 μm, a height ~ 100 μm) that swells at its tip to form a multinucleate vesicle (a diameter ~ 10 μm). Two layers of sterigmata, the metulae and phialides, form sequentially from the vesicle. Each vesicle produces about 60 metulae, and each metula buds twice to produce two phialides (Fig. 1.1a). Both metulae and phialides are uninucleate (Mims, 1971; Oliver, 1972). Chains of conidia emerge from each phialide by repeated mitosis, and it was estimated that more than 10,000 conidia can be generated from a single conidiophore. The conidia are generated basipetally, with

the youngest conidium emerging closest to the phialide apex (Adams et al., 1998; Cole, 1986; Ni et al., 2010).

An investigation of poly(A) RNA sequences distributed over three stages of development, vegetative hyphae, conidiating cultures and conidia using complementary DNA (cDNA) hybridization experiments reveals 700 ~ 1,100 diverse and new sequences (about 20% of poly(A) RNA mass) are transcribed during conidiation in *A. nidulans*. The implication of this result is a specific set of genes are required to mediate conidiation in *A. nidulans* (Timberlake, 1980).

Clutterbuck generated a set of conidiation mutants using forward genetics. The mutants do not have defects on vegetative growth, rather they exhibit abnormal conidiophore morphology and/or the number of conidia produced. Major genes corresponding to the mutant phenotype are characterized, and genetic relationship among those genes are well understood due to through epistatic analysis (Adams et al., 1988; Clutterbuck, 1969). The central genetic pathway of *A. nidulans* conidiation is composed of *brlA* → *abaA* → *wetA* (Fig. 1.2) (Boylan et al., 1987; Timberlake and Marshall, 1988).

BrlA is a key regulator of conidiation in *A. nidulans*. Disruption of BrlA causes 'bristle'-like aerial hyphae with no formation of vesicles, sterigmata, and conidia (Boylan et al., 1987; Clutterbuck, 1969). In contrast, over-expression of *brlA* ('bristle') causes cessation of vegetative growth, and formation of conidia directly from hyphae in submerged culture. Moreover, over-expression of *brlA* induces the expression of *abaA* and *wetA* in the central genetic pathway (Adams and Timberlake, 1990; Adams et al.,

1998). *brlA* encodes two transcripts, *brlAα* and *brlAβ* (2.1 kb and 2.5 kb, respectively), and both transcripts are required for normal conidiophore morphology and conidia formation (Prade and Timberlake, 1993). BrlA is a C_2H_2 zinc finger transcription factor, and expressed 10 hr post induction, which corresponds to vesicle formation (Adams et al., 1988). In addition, Aguirre et al., demonstrate that BrlA::β-Gal is expressed in vesicles, sterigmata, and immature conidia, but not in vegetative hyphae or mature conidia (Aguirre, 1993).

AbaA is a characterized ATTS/TEA transcription factor (Andrianopoulos and Timberlake, 1994). A loss of function mutant of *abaA* ('abacus') in *A. nidulans* produces conidiophores bearing 'abacus'-like sterigmata with no conidia formation. It has been suggested that a primary function of AbaA is in regulating phialide differentiation. For example, transcription of *abaA* initiates 15 h post induction, which corresponds to the time phialides form (Boylan et al., 1987). Moreover, ultra-structure analysis using transmission electron microscopy (TEM) also shows that the abnormally differentiated sterigmata do not have an extra layer found in phialides, which suggests that the abacus-like structures are similar to metulae rather than phialides (Sewall et al., 1990a). Over-expression of *abaA* in vegetative hyphae activates expression of *brlA* and *wetA*, but causes cessation of vegetative growth and vacuolization without inducing conidia formation (Aguirre, 1993; Mirabito et al., 1989).

A loss of function mutant of *wetA* ('wet-white') in *A. nidulans* produces conidia undergoing autolysis, which results in formation of water droplets in conidiophores (Clutterbuck, 1969). A mature conidium has four layers of cell wall (C1 ~ C4), and the

wetA mutant lacks the inner layer C4, which suggests that WetA is required for conidia maturation in *A. nidulans* (Sewall et al., 1990b). Transcription of *wetA* initiates 12 h post induction, which corresponds to a conidiation initiation (Mirabito et al., 1989). It has been suggested that WetA serves an important function in spore-specific gene expressions. For example, over-expression of *wetA* inhibits vegetative growth and induces expression of spore-specific genes including some genes in a SpoC1 gene cluster (a collection of genes selectively expressed in spores, but not in vegetative hyphae). However, expression of *brlA* and *abaA* are not activated by over-expression of *wetA* (Marshall and Timberlake, 1991; Timberlake and Barnard, 1981).

StuA and MedA are developmental modifiers to regulate cell differentiation in conidiophores. Deletion of *stuA* ('stunted') causes formation of conidiophores with shortened aerial stalks, and conidia production directly from either vesicles or metulae. The *medA* ('medusa') mutant produces reiterated or branched sterigmata, and occasionally secondary conidiophores. Despite their defective conidiophore morphology, the *stuA* and *medA* mutants are able to produce viable conidia (Clutterbuck, 1969). StuA and MedA are involved in spatial and temporal expression of the key regulator *brlA*, respectively (Aguirre, 1993; Busby et al., 1996; Miller et al., 1992). Details for my studies of StuA and MedA are described in Chapter II and III, respectively.

Upstream activators of the conidiation regulatory pathway are termed 'fluffy' genes, because mutations in the genes led to cotton-like vegetative mass on the colonies. There are six genes (*fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*; *flb* stands for 'fluffy low *brlA* expression') involved in activation of *brlA* (Adams et al., 1992; Wieser et al., 1994). The

favored hypothesis is that FluG is an extracellular molecule required for inducing conidiation. For example, conidiation does not take place in Δ*fluG*, but is restored when cultured next to wild type colony. The *fluG* over-expression strain can produce conidiophores in liquid submerged culture, and over-expression of *fluG* is sufficient to activate *brlA* expression (D'Souza et al., 2001). FluG contains a prokaryotic glutamine synthetase I domain in the C-terminal half, and an early nodulin gene (MtN6 in *Medicago truncatula*) -like region in the N-terminal half (Lee and Adams, 1994a; Mathis et al., 1999). Function of FluG is required to activate expression of *flbB*, *flbC*, *flbD*, *flbE*, and *brlA* to initiate conidiation (Adams et al., 1988; Lee and Adams, 1996; Wieser and Adams, 1995).

FlbA is a regulator of G protein signaling (RGS) protein (Yu et al., 1996), and a loss of function mutant of *flbA* forms an aconidial colony, which undergoes autolysis as the colony matures (Wieser et al., 1994). Over-expression of *flbA* in vegetative cells induces misscheduled *brlA* expression and premature initiation of conidiation, which suggests that FlbA is critical for activation of *brlA* (Lee and Adams, 1994b).

Mutations in other fluffy genes including *flbB*, *flbC*, *flbD*, or *flbE* leads to delayed conidiation (Wieser et al., 1994). Taken together, the results of these genetic analyses reveal the order of the upstream genes is *fluG* → *flbE* → *flbD* → *flbB* (Lee and Adams, 1996; Wieser and Adams, 1995). *flbC* and *flbD* function to activate *brlA* in the independent genetic pathway. For example, a double mutant of *flbC* and *flbD* shows additive defects in conidiation (Wieser and Adams, 1995). FlbB is a putative basic leucine zipper (b-ZIP) transcription factor, and FlbB::GFP localizes in cytoplasm in

early vegetative cell, whereas it localizes in nuclei at cell apex as it matures. *flbB* expression occurs during vegetative growth, decreases as conidiation initiates, and resumes 12 h post induction, which suggests that timely expression of *flbB* is critical for conidiation (Etxebeste et al., 2008). FlbC and FlbD are a C_2H_2 zinc finger and Myb-like transcription factors, respectively. Expression of *flbC* is detected in all developmental stages, with a higher level in vegetative growth, early conidiation, and late sexual cycle. Over-expression of *flbC* induces *brlA* expression, and causes restricted hyphal growth without conidiophore formation. In addition, FlbC::GFP localizes in nuclei in vegetative hyphae and all tissue types of a conidiophore, but not in conidia (Kwon et al., 2010). *flbD* is constitutively expressed throughout the life cycle, and the over-expressed *flbD* strain can form conidiophores in liquid submerged culture (Wieser and Adams, 1995). Together, these upstream regulators are involved in initiation of conidiation probably via transcriptional regulation of *brlA* expression.

CONIDIATION OF *Neurospora crassa*

Asexual development in *N. crassa* includes production of multinucleate macroconidia and uninucleate microconidia. Macroconidiation follows a blastic, and acropetal developmental pattern, while microconidiation produces phialidic spores (Maheshwari, 1999). Macroconidiation proceeds by transition from apical extension of aerial hyphae to budding growth, which results in the formation of short proconidial chains, termed minor constriction chains (Fig. 1.1b). Length of interconidial junctions is similar to the diameter of proconidia in minor constriction chains.

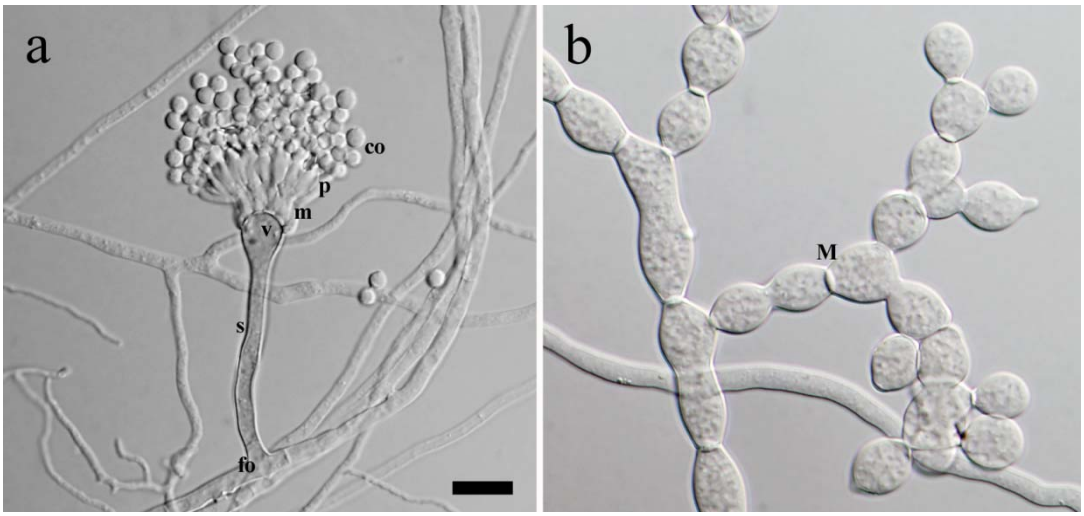

Figure 1.1 The conidiophore of *A. nidulans* and *N. crassa.*
(a) A conidiophore of *A. nidulans* wild type (A4). Conidiophore formation initiates from a specialized foot cell ('fo') from vegetative hyphae. The foot cell gives rise to an aerial stalk ('s') that swells to form a multinucleate vesicle ('v'). The metulae ('m') and phialides ('p') form sequentially from the vesicle. Chains of conidia ('co') emerge from each phialide in a basipetal manner with the youngest conidium emerging closest to the phialide apex. (b) A conidiophore of *N. crassa* wild type (74A-OR23-1VA). Macroconidiation initiates from formation of short proconidial chains, termed minor constriction chains. At later stages, the budding pattern of growth leads to formation of major constriction chains ('M') with more pronounced constrictions. Double cross-walls between the proconidia become cleaved and eventually conidia are separated from conidiophores. The conidia are produced in an acropetal manner, with the youngest conidium emerging closest to the conidium at the tip. Scale bar = 10 μm.

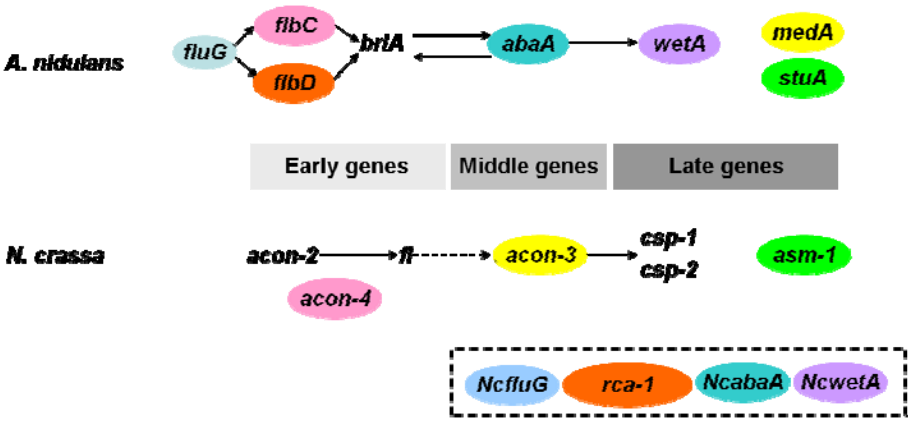

Figure 1.2 Genetic pathways of conidiation regulators in *A. nidulans* and their orthologs in *N. crassa.*
(Top) Conidiation of *A. nidulans*. *brlA* is the key regulator of *A. nidulans* conidiation. *fluG*, *flbC*, and *flbD* are upstream regulators to activate *brlA* expression for conidiation initiation. *brlA* → *abaA* → *wetA* comprises the central conidiation regulatory pathway in *A. nidulans*. *medA* are *stuA* are developmental modifiers and the mutants of *medA* and *stuA* are oligosporogenous with decreased number of conidia produced compare to wild type. (Bottom) Macroconidiation of *N. crassa*, and orthologs to the *A. nidulans* conidiation regulators. The orthologs were characterized with amino acid sequences using BLASTP program. *fl* is the key regulator of *N. crassa* conidiation. *acon-2* and *acon-4* are upstream regulators to initiate conidiation. The dashed line between *fl* and *acon-3* indicates the order of *fl* and *acon-3* are not characterized. *csp-1* and *csp-2* are involved in conidiation separation in the later developmental stage. The *asm-1* mutant displays abnormal conidiophore morphology, but is able to produce macroconidia. Genes surrounded by a dashed box represent *N. crassa* orthologs that do not exhibit discernable macroconidiation phenotypes when knocked out in *N. crassa*. Orthology of the genes between *A. nidulans* and *N. crassa* is indicated by an identical color. Note that *brlA* and *fl* are not homologous to each other. There are no homologs of *brlA* and *fl* found in *N. crassa* and *A. nidulans*, respectively.

At later stages, the budding pattern of growth leads to formation of major constriction chains with more pronounced constrictions (Fig. 1.1b). Double cross-walls between the proconidia become cleaved and eventually conidia separate from conidiophores. The conidia are produced acropetally, with the youngest conidium emerging closest to the conidium at the tip (Bailey and Ebbole, 1998; Springer and Yanofsky, 1989).

Arthroconidia are another type of conidia in *N. crassa*. They constitute approximately 1% of the total conidial mass, and first appear ~10 hr after conidiation induction. Arthroconidia are produced in a thallic-arthric manner. For example, the hyphae that give rise to a conidiophore form extra cross-walls with a distance of ~ 10 μm. Then the cross-walls become thickened and separated as they mature, which results in the disarticulated hyphal segments termed arthroconidia (Cole, 1986; Springer and Yanofsky, 1989).

Springer and Yanofsky (1989) provide a summary of *N. crassa* mutants generated by reverse genetics, that have defects in macroconidiation. There are 22 genetic loci listed in their review of *N. crassa* conidiation study (Springer and Yanofsky, 1989), but only 5 of them (*acon-2*, *acon-3*, *csp-1*, *csp-2*, and *fl*) are discussed here. The *acon-2* ('aconidiate-2') mutant produces aerial hyphae without minor constriction chain formation. *acon-2* is a temperature-sensitive mutant conidiating normally at 25°C, but it is aconidial at 34°C. Culturing the mutant on carbon-starvation media results in an aconidiate phenotype at either temperature (25°C or 34°C) (Matsuyama et al., 1974). Both *acon-3* ('aconidiate-3') and *fl* ('fluffy') mutants are able to produce minor

constriction chains, but are blocked in production of major constriction chains. Compared to the *acon-3* mutant, a *fl* mutant has a more severe defect in conidiation. The minor constriction chains of the *acon-3* mutant produce septa and occasionally revert to hyphal growth, whereas, the *fl* mutant produces conidiophores budding only a few times, and does not septate (Matsuyama et al., 1974). Two other mutants, *csp-1* and *csp-2* ('conidial-separation' -1 and -2) form normal conidiophores and conidia, but the conidia do not separate from the conidiophores. Thus, the *csp-1* and *csp-2* mutants may have defective autolytic activity on their interconidial junctions (Selitrennikoff et al., 1974).

In contrast, studies of double mutants reveal a genetic pathway for macroconidiation in *N. crassa* (Davis, 1970; Perkins, 1966). For example, *acon-2*; *acon-3* and *acon-2*; *fl* double mutants show *acon-2* – like phenotypes at the non-permissive temperature (34°C), which suggests that *acon-2* functions before *acon-3* and *fl* to mediate macro-conidiation. In contrast, the order of *acon-3* and *fl* in the genetic pathway is less clear. The double mutant of *acon-3*; *fl* produces minor constriction chains without proceeding to formation of major constriction chains, which is consistent with the phenotypes of the *acon-3* and *fl* mutants (Fig. 1.2). However, the number of minor constriction chains formed in the double mutant is much lower than in either the *acon-3* or *fl* mutant (Davis, 1970; Perkins, 1966).

CROSS-SPECIES COMPLEMENTATION OF THE CONIDIATION MUTANTS IN ASCOMYCETES

Expression of *N. crassa flbD* ortholog, *rca-1* (regulator of conidiation in *Aspergillus*) complements defective conidiation in an *A. nidulans flbD* mutant (Shen et al., 1998). A loss of function mutation in FlbD causes production of 'fluffy'-like hyphal mass and a delay in conidia formation. In contrast, Δ*rca-1* displays no detectable defects in either macoconidiation and microconidiation in *N. crassa*, which shows that despite the distinct phenotypic effects in individual species, an ortholog can restore biochemical activity for conidiation in a distantly related species (Shen et al., 1998).

AbaA and StuA have similar phenotypic effects in *A. nidulans* and *Penicillium marneffei* and cross-species complementation analysis suggests conserved function. In *P. marneffei*, Δ*abaA* is aconidial and produces reiterating cells with no phialides formed. Expression of *A. nidulans abaA* using its native promoter complements defective conidiation in *P. marneffei* Δ*abaA*, which suggests that AbaA proteins share a conserved biochemical function between the two species (Borneman et al., 2000). In *A. nidulans*, Δ*stuA* produces shortened stalks, and conidia are produced from either a vesicle or a metulae. Δ*stuA* in *P. marneffei* displays similar phenotype. For example, *P. marneffei* Δ*stuA* produces no metulae and phialides, and conidia are produced directly from a stalk. Expression of *P. marneffei stuA* using its native promoter complements defective conidiation in *A. nidulans* Δ*stuA*, which suggests that StuA family proteins have conserved biochemical function in these species as was found for the AbaA family proteins (Borneman et al., 2002).

HYPOTHESIS

The ascomycetes represent the largest phylum of the kingdom Fungi, and undergo asexual reproduction called conidiation. I hypothesize that a conidiation regulatory pathway was present in the ancestral species, and became specialized to lead to the morphological and functional diversity found in the extant species. If the conidiation pathway in the last common ancestor included conidiation regulatory genes found in the species of today, we expect to observe *N. crassa* orthologs to these genes (i) would be involved in conidiation in *N. crassa*, and (ii) would complement defective conidiation in the *A. nidulans* mutants.

In this study, *N. crassa* orthologs corresponding to 7 *A. nidulans* conidiation regulators (*fluG*, *flbC*, *flbD*, *abaA*, *wetA*, *medA*, and *stuA*) were identified using the BLASTP program. The seven genes and the corresponding *N. crassa* orthologs were divided into three groups – early, middle, and late genes (Fig. 1.2) depending on the developmental stage and associated gene functions. For my dissertation, I studied three genes – *medA* (Chapter II), and *abaA* and *stuA* (Chapter III), however, the results for parallel studies of other genes are included in the CONCLUSION section to provide context.

The goal of this project is to understand the evolution of conidiation machinery in ascomycetes. Specific objectives are (i) to characterize function of known conidiation regulator further in *A. nidulans* (mutant phenotype, subcellular localization of gene products, etc.), (ii) to assess the biochemical function of orthologous *N. crassa* genes in

the context of the *A. nidulans* mutant background (cross-species complementation), and (iii) to define evolutionary patterns based on the result from Objective (ii).

CHAPTER II

acon-3, THE *Neurospora crassa* ORTHOLOG OF THE DEVELOPMENTAL MODIFIER, *medA*, COMPLEMENTS THE CONIDIATION DEFECT OF THE *Aspergillus nidulans* MUTANT[*]

SUMMARY

Aspergillus nidulans and *Neurospora crassa* are ascomycetes that produce asexual spores through morphologically distinct processes. MedA, a protein with unknown function, is required for normal asexual and sexual development in *A. nidulans*. We determined that the *N. crassa* ortholog of *medA* is *acon-3*, a gene required for early conidiophore development and female fertility. To test hypotheses about the evolutionary origins of asexual development in distinct fungal lineages it is important to understand the degree of conservation of developmental regulators. The amino acid sequences of *A. nidulans* MedA and *N. crassa* ACON-3 shared 37% identity and 51% similarity. *acon-3* is induced at late time points of conidiation. In contrast, *medA* is constitutively expressed and MedA protein localizes to nuclei in all tissue types. Nonetheless, expression of *acon-3* using its native promoter complemented the conidiation defects of the *A. nidulans* Δ*medA* and *medA15* mutants. We conclude that the biochemical activity of the *medA* orthologs is conserved for conidiation.

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[*] This chapter is reprinted with permission from "*acon-3*, the *Neurospora crassa* orthologs of the developmenta modifier, *medA*, complements the conidiation defect of the *Aspergillus nidulans* mutant" by Chung, D.-W., Greenwald, C., Upadhyay, S., Ding, S., Wilkinson, H. H., Ebbole, D. J. and Shaw, B. D. 2011. Fungal Genet Biol. 48: 370-376.

INTRODUCTION

Conidia are asexual spores produced by fungi including important species that impact agriculture, industry, and medicine (Adams et al., 1998; Ebbole, 2010). Conidia serve as propagules for infection of plants and animals. Moreover, conidiation is a critical reproductive mode in more than 15,000 described fungal species not known to undergo sexual development (Cole, 1986).

Aspergillus nidulans and *Neurospora crassa* are model organisms for molecular genetic studies. They are estimated to have diverged at least 215 million years ago (Taylor and Berbee, 2006), and morphological events in conidiation have been extensively documented. The developmental pathways for these two fungi are fundamentally different in their morphogenesis. Conidiophore formation in *A. nidulans* initiates from a specialized foot cell with a thickened cell wall. The foot cell gives rise to an aerial stalk that swells at its tip to form a vesicle. Two layers of sterigmata, the metulae and phialides, form sequentially from the vesicle, and chains of conidia emerge from each phialide. The conidia are generated basipetally, with the youngest conidium emerging closest to the phialide apex (Adams et al., 1998; Cole, 1986; Ni et al., 2010). Asexual development in *N. crassa* includes production of multinucleate macroconidia and uninucleate microconidia. Macroconidiation follows a blastic, and acropetal developmental pattern, while microconidiation produces phialidic spores (Maheshwari, 1999). Macroconidiation proceeds by transition from apical extension of aerial hyphae to budding growth, which results in the formation of short proconidial chains, termed minor constriction chains. At later stages, the budding pattern of growth leads to

formation of major constriction chains with more pronounced constrictions. Double cross-walls between the proconidia become cleaved and eventually conidia are separated from conidiophores (Bailey and Ebbole, 1998; Springer and Yanofsky, 1989).

Clutterbuck generated *A. nidulans* mutants that showed defects in conidiation, but not in vegetative growth (Clutterbuck, 1969). A number of later studies with the conidial mutants proposed a central regulatory pathway of *A. nidulans* development. Three genes, *brlA* (bristle), *abaA* (abacus), and *wetA* (wet-white) were identified as central regulators governing conidiation. BrlA in particular plays a key role in *A. nidulans* conidiation, and the *brlA* null mutant failed to transition from the conidiophore stalk to vesicle formation (Timberlake, 1990).

Important regulatory genes have been identified for both fungi. Fluffy (*fl)*, the key regulatory gene in *N. crassa* conidiation (Bailey and Ebbole, 1998; Springer and Yanofsky, 1989), plays an analogous role to *brlA* in *A. nidulans* (Boylan et al., 1987), yet they are not homologs. Furthermore, the *A. nidulans* genome does not contain an obvious ortholog of *fl* nor does the *N. crassa* genome contain an obvious ortholog of *brlA*. Orthologs to *fl* do appear in the Sordariomycetes *Sordaria macrospora* and *Podospora anserina*, however, it is noteworthy that these fungi do not produce macroconidia. Orthologs to *brlA* are found in the Eurotiomycetes *Aspergillus* sp., and *Penicillium* sp.. Considered alone, the lineage specificity of *brlA* and *fl* suggests that regulators of conidiation may not be conserved in different lineages of fungi and that these developmental pathways may have evolved independently.

Conversely, another gene that regulates conidiation in *A. nidulans, flbD* (fluffy low *brlA*) has an ortholog in *N. crassa, rca-1* (regulator of conidiation in *Aspergillus*). Transformation with a DNA fragment containing the *N. crassa rca-1* gene complemented the conidiation phenotype of the *A. nidulans flbD* mutant (Shen et al., 1998). The FlbD and RCA-1 proteins display 75% similarity in the putative DNA-binding domain and 42% similarity in other regions. Deletion of *rca-1* does not affect macroconidiation, microconidiation, or ascospore formation in *N. crassa*. Thus, this is an example where the ortholog to a regulator of conidiation does restore the function in conidiation, however, the role of *rca-1* in *N. crassa* is distinct from the role of *flbD* in *A. nidulans* (Shen et al., 1998). Therefore, during the divergence of these fungal lineages there has been either a gain of the function in conidiation for *flbD* or loss of the conidiation function for *rca-1*.

MedA has been characterized as a developmental modifier in *A. nidulans* because it coordinates temporal and spatial organization of the conidiophores. Disruption of *medA* (medusa) alters the temporal expression of *brlA*, and the *medA* mutant alleles produce abnormal conidiophores with multiple layers of sterigmata and occasionally secondary conidiophores (Busby et al., 1996; Clutterbuck, 1969). Although the *medA* orthologs in other filamentous fungi, including *Magnaporthe oryzae* and *Fusarium oxysporum,* have been shown to play a role in conidiation (Lau and Hamer, 1998; Ohara et al., 2004) the biochemical function of MedA in conidial development is not well understood.

Here I studied the functional conservation of the *medA* orthologs in *A. nidulans* (ANID_06230.1) and *N. crassa* (NCU07617.4). Based on our results the *N. crassa* ortholog to *medA* is *acon-3* (*acon*idiate-*3*). I have expressed *acon-3* in *A. nidulans medA* mutants (*ΔmedA* and *medA15*), and analyzed conidiophore morphology and conidia production compared to the *medA* mutants. Transcript expression was compared between the organisms and the temporal aspects of expression and localization were monitored in *A. nidulans* using a green fluorescence protein (GFP) fusion in live cells.

MATERIALS AND METHODS

Strains, media, and growth conditions

A list of *A. nidulans* strains, and plasmids and constructs used in this study is shown in Table 2.1 and Table 2.2, respectively. Minimal medium (MM) and complete medium (CM) for growing *A. nidulans* strains were prepared with appropriate supplements as described (http://www.fgsc.net). In physiological studies, strains were cultured at 28 °C unless otherwise indicated. All reagents for media, supplements, and buffers used were purchased from Sigma (St. Louis, MO) unless otherwise indicated. All plasmids were stored and amplified in *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA).

Conidia production studies were carried out on MM with appropriate supplements. Ten microliters of sterile water containing 10^6 conidia were placed in the center of a solid MM plate, and incubated at 28 °C for 5 days. Then, conidia were collected with sterile water and the number of conidia was counted using a hemocytometer. This experiment was performed in triplicate.

Table 2.1 Strains used in this study (*medA*)

Strains	Genotype	Source
A4	*A. nidulans* wild type	FGSC*
A586	*biA1*; *medA15*; *veA1*	FGSC*
A773	*pyrG89*; *pyroA4*;*wA3*; *veA1*	FGSC*
PW1	*biA1*; *argB2*; *methG1*;*veA1*	Goc et al. (1987)
TN02A25	*pyrG89*; *argB2*; *pabaB22*; *nkuA*::*argB*; *riboB2*; *veA1*	Nayak et al. (2006)
ASL91	*pyrG89*; *argB2*; *wA3*; *veA1*	This study
Asum3	*biA1*; *medA15*; *argB2*; *pyrG89*; *wA3*; *veA1*	This study
Δ*medA*	*ΔmedA*; *pabaB22*; *riboB2*; *veA1*	This study
TDC1.23 / TDC1.29	*medA15*; *argB2*; *pyrG89*; *wA3*; *veA1*; *acon-3*::*argB*	This study
TDC3.38	*pyrG89*; *pyroA4*; *wA3*; *veA1*; *medA*::*eGFP*; *A. fumigatus pyrG* (*AfpyrG*)	This study
TDC6.14 / TDC6.19	*biA1*; *medA15*; *argB2*; *pyrG89*; *veA1*; *medA*:: *argB*	This study
RDC20.10	*acon-3*::*argB*; *methG1*;*wA3*;*veA1*	This study
RDC21.1	*ΔmedA*;*acon-3*::*argB*; *riboB2*; *veA1*	This study
RDC22.3	*ΔmedA*;*medA*::*argB*; *riboB2*; *veA1*	This study
74A-OR23-1VA	*N. crassa* wild type	FGSC

* Fungal Genetics Stock Center (McCluskey, 2003)

Table 2.2 Plasmid/construct used in this study (*medA*)

Plasmids	Characteristics	Source
pGEM-T easy	TA cloning vector	Promega
pENTR	Entry vector for GATEWAY cloning	Invitrogen
pFN03	*GA5*::*eGFP*; *A. fumigatus pyrG* (*AfpyrG*)	Yang *et al*., 2004
pCG1	pCB1004; *acon-3*	This study
pDC1.1	*medA* coding region with 1.5 kb up- and 0.5 kb downstream sequence; *argB*	This study
pDC2.1	*acon-3* coding region with 1.6 kb up-and 1.5 kb downstream sequence; *argB*	This study
Δ*medA* cassette	ANID_06230.1 coding sequence with 774 bp up- and 685 bp downstream sequence; *AfpyrG*	FGSC*
pDC3.1	pGEM-1.3kb of *medA* coding sequence *GA5*:: *eGFP*; *AfpyrG*	This study

* Program Project Grant GM068087

Identification of orthologs

To find the putative ortholog of *A. nidulans medA*, we used a 705 amino acid sequence of *A. nidulans* MedA as a query sequence against the *N. crassa* genome (http://www.broadinstitute.org/annotation/genome/neurospora) using the BLASTP program. The 693 amino acid sequence of NCU07617.4 was the sole candidate for orthology to *medA*, and this result was confirmed by reciprocal BLAST against the *A. nidulans* database. Additional putative orthologs of *A. nidulans medA* in other fungal species were identified using the same approach. Sequences used for the bi-directional comparison of the putative orthologs in *M. oryzae*, *F. oxysporum*, *Botrytis cinerea*, *Coccidioides immitis*, *Sclerotinia sclerotiorum*, *Ustilago maydis*, and *Cryptococcus neoformans* were obtained from the Fungal Genome Initiative database at the Broad Institute (http://www.broadinstitute.org/science/projects/fungal-genome-initiative). Sequence of the *Penicillium marneffei medA* ortholog (XP_002147078) was obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and sequence of the *Trichoderma virens* ortholog (Trive1|34415) was obtained from the DOE Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Trive1/Trive1.home.html). Sequence alignment was performed using the ClustalW program online at EMBL-EBI (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and visualized with GeneDoc software (Nicholas et al., 1997).

Molecular cloning procedures

pDC2.1, used to complement the *A. nidulans medA15* mutant and *ΔmedA* with *N. crassa acon-3*, was designed to express *acon-3* using the native *N. crassa* promoter. Oligonucleotides used in this study are summarized in Table 2.3. A DNA fragment of 5,426 bp including the 2,359 bp coding region, 1,490 bp of upstream sequence and 1,577 bp of downstream sequence of *acon-3* was amplified using the primers NcmedAF and NcmedAR. The PCR product was digested using *Xba*I, and ligated into the *Xba*I digested pTA-argB vector, that carried the *A. nidulans argB* sequence as a selectable marker.

The plasmid pDC1.1 contained a 4,179 bp clone of *A. nidulans medA* including the 2,084 bp coding region, 1,659 bp upstream and 436 bp downstream of the *medA* coding region was amplified using the primers AnmedAF-SpeI and AnmedAR-SpeI. The PCR product was digested using *Spe*I, and ligated into *Spe*I digested pTA-argB vector.

Strain A586 was crossed to strain ASL91 to generate strain Asum3 (*argB2*; *pyrG89*; *medA15*). The plasmids, pDC1.1, and pDC2.1 were transformed into Asum3 using the protoplast transformation method. This resulted in generation of pDC1.1 transformants TDC6.14 and TDC6.19, and pDC2.1 transformants TDC1.23 and TDC1.29. A single copy insertion of the plasmid pDC2.1 was verified by PCR and Southern blot analysis.

Table 2.3 Oligonucleotides used in this study (*medA*)

Designation	Sequence (5'→ 3')
AN6542F actin	CACCATGGAAGGTAAGGTTT
ActGateR	GAAGCACTTGCGGTGGACGA
brlA5F	CCGATCGTCTGACCGTTGAAGTT
brlA3R	CCAGCTTTGACCCGTAGATAGGA
NcmedAAF3	CACCATGTCGTCCCTCAAGTTCCA
NcmedAAR3	TTAGGCGCGGTGTACATCC
NcmedAF	GGCCCGTCTTTCTAGACCCCCCCTG
NcmedAR	GATC CGATCTAGATGGAGATGC
AnmedAF-SpeI	CCTTCAGC AACTAGTCGTCATCAG
AnmedAR-SpeI	TCCACACTTTACTAGTCCAATAATGG
MedA-TOPOF	CACCATGTCTGGTTACCAGAGAACC
MedA-TOPOR	GTGTCCCTGG GGGTAATCAGACT
MedAGFP-Fu1	CAACTC TGCAGGTGGGCGTTTAT
MedAGFP-Fu2	GTGTCCCTGGGGGTAATC AGACT
MedAGFP-Fu5	CGAACCGAGCTCGTGTCTCTG
MedAGFP-Fu6	ACGCTTCTCCCGTTTGACCTTC
MedAGFP-Fnest	CCTTACTCGCCATATCTGCAGCC
MedAGFP-Rnest	ACGGATGGAAGTCACCAAGAAGAC
NcMedA-SF	CCTCAGACGAGCTCCAACATCAC
NcMedA-SR	GGATCCCCTGTTGTCATACACGC
Acon3-C1	TGTCGACCTTCTAGCACACCCAAT
Acon3-C2	AGTCGAGTCGCATCGTGATT
Acon3-C3	TGACAAGGTGAAAGCACAGC
Acon3KI-5	GATGTACACCGCGCCTAACGAACCGAGCTCGTGTCT
medAKO-ScR	CACCCATGCGGCATAATTATACA
Acon3KI-seq1	GTCATTTCCACTCTTCTCACTCGC

To generate the Δ*medA* strain, the *medA* knock-out cassette from the FGSC (McCluskey, 2003; Program Project Grant GM068087) was transformed into *A. nidulans* wild type TN02A25 using the protoplast transformation method. This resulted in generation of a transformant, Δ*medA*. Homologous replacement of *medA* with *Aspergillus fumigatus pyrG* was verified by PCR and Southern blot analysis. To generate strain RDC20.10, TDC1.23 was crossed to *A. nidulans* wild type PW1, and the cross progeny were screened by PCR. Strain RDC21.1 and RDC22.3 were generated by crossing Δ*medA* to RDC20.10 and TDC6.14, respectively.

Identification of *acon-3*

The NCU07617.4 locus was amplified from both *N. crassa acon*-3 (FGSC #5074) and the wild type strain 74-OR23-1VA using FideliTaq™ DNA polymerase. Primers Acon3-C2 and Acon3-C3 were used to amplify the locus from *N. crassa acon-3*. This 3,534 bp PCR product contained 755 bp upstream of the coding region and 419 bp downstream of the coding region. Primers Acon3-C1 and Acon3-C2 were used to amplify the locus from the *N. crassa* wild type strain. This 4,897 bp PCR product contained 2,118 bp upstream of the coding region and 419 bp downstream of the coding region. The PCR products were then inserted into a pCR4-TOPO vector using the manufacturers protocol (Invitrogen, Carlsbad, CA), and transformed into chemically competent *E. coli*. In both cases the cloned inserts from two independent PCR reactions were sequenced with 4X coverage. The clone containing the PCR product from wild type was digested with *Eco*RI, and the insert was ligated into vector pCB1004 (Carroll et

al., 1994) to allow a test for complementation of the *acon-3* mutant. This resulted in plasmid pCG1.

Transformable spheroplasts of *N. crassa acon-3* (FGSC #5074) were generated according to the previously described protocol (Royer and Yamashiro, 1992). Briefly, *acon-3* was grown in Vogel's minimal medium. After each day of growth in a shaking incubator (34 °C, 200 rpm), the mycelial suspension was blended, and a portion (30 mL) was then used to inoculate the next day's growth in 100 mL of fresh liquid medium (130 mL total). After growth, spheroplasts were generated and transformed with 1 μg of DNA from pCG1 (pCB1004;NCU07617.4).

Top agar was added to the transformed spheroplasts and then overlaid on top of FGS medium containing 250 μg/mL hygromycin. Transformants were then transferred to tubes containing Vogel's medium and 250 μg/mL hygromycin and subsequently screened for presence or absence of conidia. Conidiating cultures were subjected to serial transfers on FGS plates to favor homokaryons (Ebbole and Sachs, 1990).

GFP tagging of *A. nidulans* MedA

To generate strains expressing MedA::GFP, we integrated a *medA::eGFP* fusion replacing the native *medA* locus. The construct was created using the fusion PCR protocol and vectors previously described (Yang et al., 2004). Briefly, a 1.3 kb fragment of the *medA* coding sequence and a 1,080 bp downstream sequence were PCR-amplified using the primers MedAGFP-Fu1, MedAGFP-Fu2, MedAGFP-Fu5 and MedAGFP-Fu6. These two PCR products were then fused to a 2.6 kb GA5-eGFP-AfpyrG fragment

amplified from pFN03 (Yang et al., 2004) using the primers MedAGFP-Fnest and MedAGFP-Rnest by the fusion PCR method resulting in a 4.9 kb construct for *medA::eGFP*. This final PCR product was cloned into pGEM-T easy vector to generate pDC3.1. pDC3.1 was transformed into the strain, A773, and the transformants were screened for the presence of GFP by PCR and microscopic observation. Homologous recombination of the *medA::eGFP* cassette at the *medA* locus was verified by Southern blot analysis.

Southern and northern blot analysis

Southern and northern blot analyses were conducted following standard procedures (Sambrook et al., 1989). To verify homologous replacement of *medA* with *A. fumigatus pyrG*, genomic DNA of Δ*medA* and *A. nidulans* wild type (A4) was isolated from mycelia, and digested using *XhoI*. The probe was generated using a 368 bp PCR fragment amplified with primers acon3KI-5 and medAKO-ScR. To verify a single copy insertion of *N. crassa acon-3*, genomic DNA of the transformants (TDC1.23, and TDC1.29) and *N. crassa* wild type (74A-OR23-1VA) was isolated from mycelia (Upadhyay and Shaw, 2006), and digested using *Sac*I and *Spe*I. The probe was generated using a 2,359 bp PCR fragment amplified with primers NcmedAAF3 and NcmedAAR3. To verify GFP-tagging of MedA at the native locus, genomic DNA of both *A. nidulans* A4 and TDC 3.38 strains was isolated from mycelia, and digested by *Pst*I and *Xho*I. The probe was made using a 1,080 bp PCR fragment amplified with primers MedAGFP-Fu5 and MedAGFP-Fu6. Production of RNA for northern blot analysis of A4, 74A-OR23-

1VA, Asum3, and TDC 1.23 involved culturing 5 x 10^6 conidia/ml in 1L of MM containing 2% glucose and 0.5% yeast extract (YG) at 28 °C shaking at 200 rpm for 24 hrs. Mycelia from 100mL of each culture was collected onto Whatmann filter paper no. 2 by vacuum filtering, and incubated on solid YG plates at 28 °C under light for 0, 4, 7, 10, 13, 16, or 24 hrs. Total RNA was extracted from mycelial pads using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The PCR products obtained with primers MedA-TOPOF and MedA-TOPOR, NcMedASF and NcMedASR, An6542F and ActGateR, and brlA5F and brlA3R were used as probes to detect expression of *medA*, *acon-3*, *A. nidulans actA*, and *brlA*, respectively.

Light microscopy

To study cell growth and visualize GFP fusion proteins, germlings or hyphae were grown on coverslips submerged in liquid MM medium. Microscopic observation and image processing were performed as previously described (Shaw and Upadhyay, 2005). To image MedA::GFP localization in conidiophores, the TDC3.38 strain was cultured on MM agar plugs overlaid with coverslips using Riddell mounts (Riddell, 1950). Observation of localization was performed as previously described (Upadhyay and Shaw, 2006).

RESULTS AND DISCUSSION

medA orthologs in filamentous fungi

Orthologs of *medA* have been studied in several filamentous fungi including *A. nidulans*, *A. fumigatus*, *F. oxysporum*, and *M. oryzae* (Busby et al., 1996; Clutterbuck, 1969; Gravelat et al., 2009; Lau and Hamer, 1998; Ohara et al., 2004). Although all the previously studied *medA* orthologs played a role in conidiation in each species, there were different phenotypic consequences. For example, *medA* mutants have altered conidiophore morphology in *A. nidulans* but in a closely related species, *A. fumigatus* (which lacks metulae), the *medA* mutant produced fewer conidia, but conidiophore morphology was normal (Gravelat et al., 2009). In *M. oryzae*, *acr1* (acropetal) mutants have dramatically altered conidiophore structure and form appressoria from only 5% of the germinated conidia, resulting in attenuated pathogenicity (Lau and Hamer, 1998). In contrast, the *F. oxysporum REN1* mutant retains pathogenicity, but its conidiophore structure is abnormal. This suggests that a role for the *medA* orthologs in conidiation is conserved. In addition, *medA* has been adapted for other developmental processes in some species (Lau and Hamer, 1998; Ohara et al., 2004).

The ortholog of *A. nidulans medA* in the *N. crassa* genome was NCU07617.4. Amino acid sequences of MedA and the *N. crassa* ortholog shared 37% identity and 51% similarity for the aligned 590 amino acids. I constructed an amino acid sequence alignment using ClustalW, and the sequence was most conserved in a C-terminal region corresponding to amino acids 351-514 of MedA (Fig. 2.1). No known protein domains

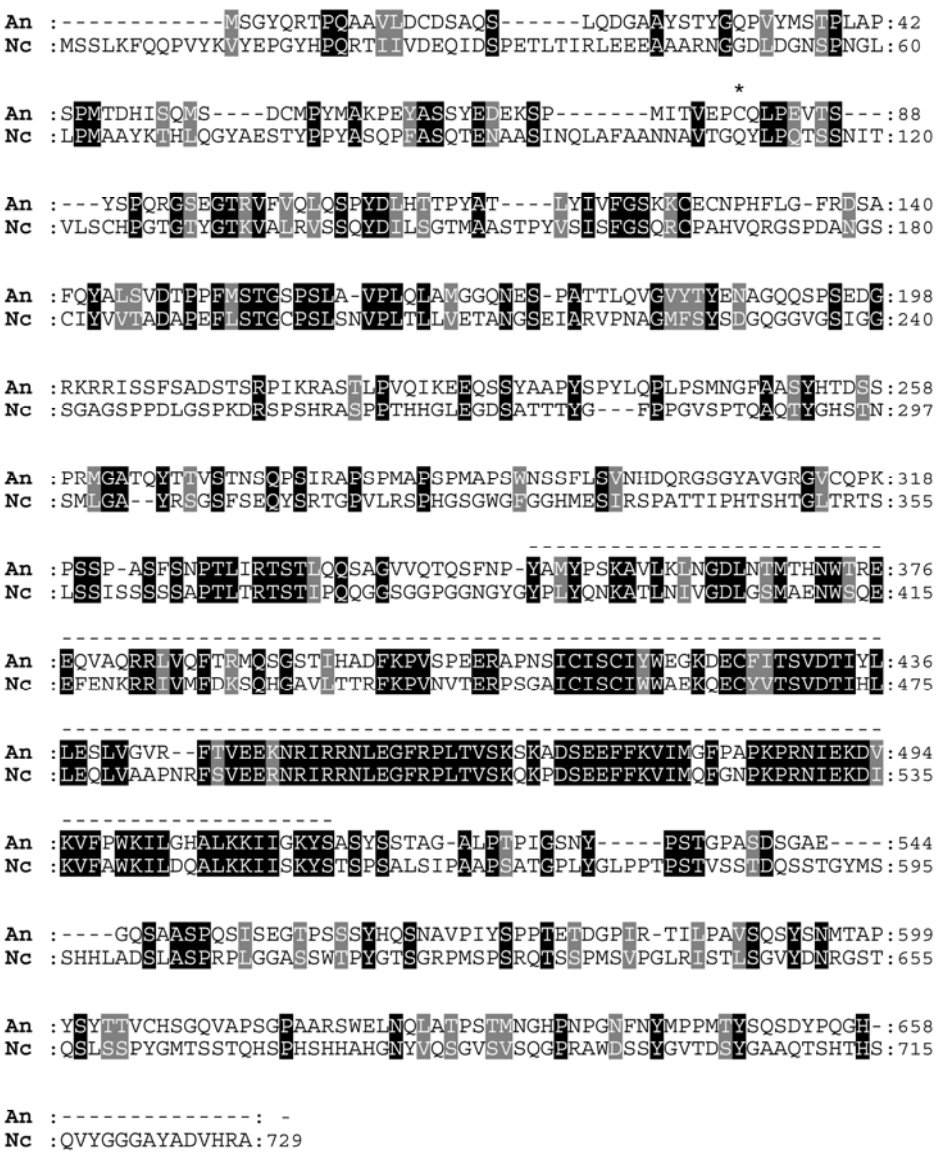

Fig. 2.1 Similarity of *A. nidulans* MedA to the *N. crassa* ortholog.
The amino acid sequence of MedA (An) was aligned with ACON-3 (Nc) using ClustalW and GeneDoc. Similar and identical residues are indicated in gray and black, respectively. Sequence similarity is intermittent across the sequence with the highest degree of conservation in a region near the C-terminus from residues 351-514 of the MedA sequence, as indicated by dashes above the sequence. No known functional domains were identifiable. The location of the *AnmedA15* point mutation (residue 81) resulting a premature stop codon (TGC to TGA) is indicated with an asterisk above the sequence.

were identified in these conserved sequences using PROSITE (de Castro et al., 2006; Hulo et al., 2006). I noted there were several stretches of basic amino acids in this region, however, these did not match consensus sequences for nuclear localization signals. Further, I examined the alignments of putative *medA* orthologs from several fungal species and the C-terminal region was remarkably conserved across the ascomycetes and basidiomycetes (Fig. 2.2). Sequence similarity outside of the C-terminal domain was limited, with a region near the N-terminus exhibiting greater conservation among the ascomycetes.

Saccharomyces cerevisiae and *Ashbya gossypii* lacked a *medA* ortholog. Other species of the Saccharomycetales, such as *Candida albicans* and *Yarrowia lipolytica*, possessed an ortholog, however, there was a large degree of sequence divergence (data not shown). *medA* orthologs were absent from the available sequences of members of the chytridiomycetes and zygomycetes.

A. nidulans medA and *N. crassa acon-3* expression and mRNA sequencing

mRNA-seq data (Illumina) from our ongoing study (unpublished) for both *A. nidulans* and *N. crassa* allowed us to define the 5' and 3' ends of the transcripts and validate the introns from the cDNA sequence for *medA* (GenBank ID: BAD02337). Based on the mRNA-seq data and alignments of the gene sequences of orthologs across fungal species, the start position of the predicted *medA* open reading frame was confirmed. However, it should be noted that this sequence is 47 amino acids shorter than

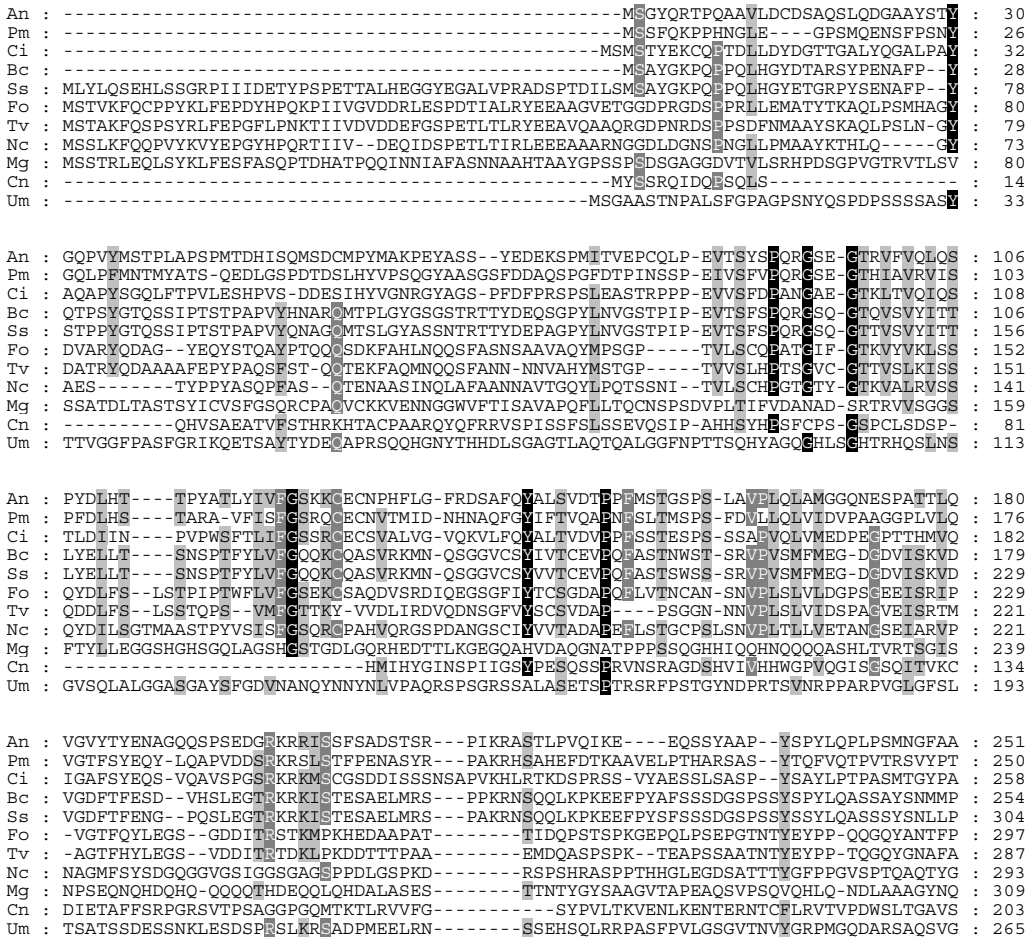

Figure 2.2 Amino acid sequence alignment for MedA orthologs from representatives of the ascomycetes and basidiomycetes.

Similar and identical residues are indicated in progressively darker shades of gray and black depending on the number of sequences containing the residue. Sequence similarity is high in the C-terminal region indicated in Figure 1. Similarity across the remaining protein sequence is intermittent, with a region near the N-terminus being more conserved in the Ascomycota. Representative species include: **An**: *A. nidulans*, **Pm**: *Penicillium marneffei*, **Ci**: *Coccidioides immitis*, **Bc**: *Botrytis cinerea*, **Sc**: *Sclerotinia sclerotiorum*, **Fo**: *Fusarium oxysporum*, **Tv**: *Trichoderma virens*, **Nc**: *Neurospora crassa*, **Mg**: *Magnaporthe oryzae*, **Cn**: *Cryptococcus neoformans*, **Um**: *Ustilago maydis*.

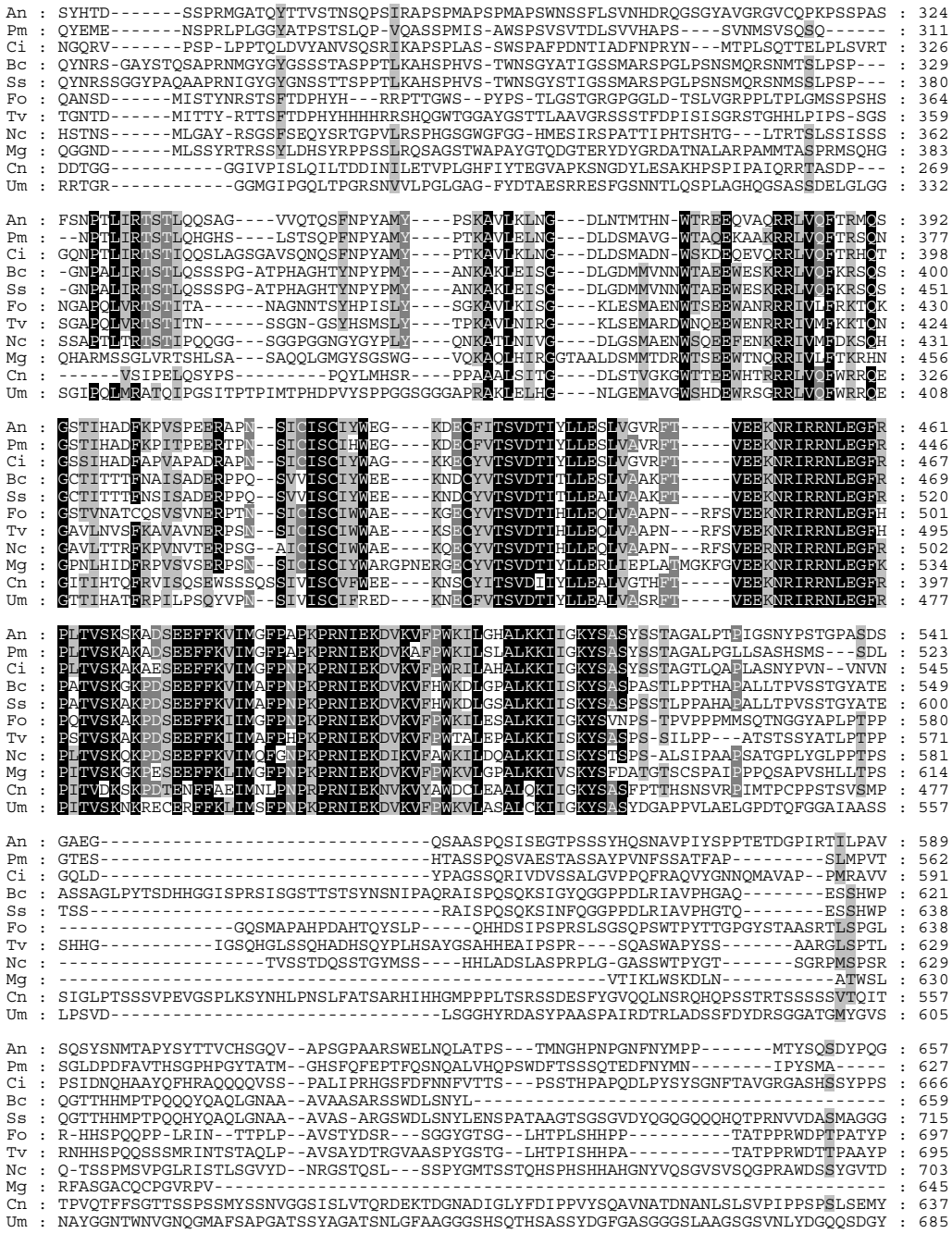

Figure 2.2 Continued.

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An : H------------------------------------------------------------ : 658
Pm : ------------------------------------------------------------ :   -
Ci : HPGGGV------------------------------------------------------- : 672
Bc : ------------------------------------------------------------ :   -
Ss : NGSRTLSSLQHQQQSQQIPRT---------------------------------------- : 736
Fo : -EGYTSLTSQT---AQPVYGAAGYAEPAPRA------------------------------ : 724
Tv : DSGYPGLTSQQPPGASSVYSTAAYGEPSHRA------------------------------ : 726
Nc : SYGAAQTSHTH----SQVYGGGAYAD-VHRA------------------------------ : 729
Mg : ------------------------------------------------------------ :   -
Cn : SAPLHYPSSSSYVQFQPPYTPSVAHVEQQQAAQYHESLRQNHDARTVYSIYPDIQGQGHGGYRNKG : 703
Um : GAATAPPHSQSQCTTSTLAAAAGDYYSSERR------------------------------ : 716
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Figure 2.2 Continued.

that of the Broad Institute annotation for ANID_06230.1. We also confirmed that the 5'UTR of *medA* is long and transcripts initiate about 2.1 kb upstream of the translation start codon. The 5'UTR of NCU07617.4 starts ~470 bp upstream of the translation start codon. The second intron of NCU07617.4 is 71 nucleotides long in our sequence analysis, which is 114 nucleotides shorter than the Broad Institute annotation for this intron and results in a protein that is 37 amino acids longer than that predicted for NCU07617.4 (GenBank ID: HM625906).

NCU07617.4 represents the *acon-3* gene

A knock-out mutant for the NCU07617.4 coding region is available from the *Neurospora* Genome Project (Colot et al., 2006). This mutant has abnormal conidiation and does not produce perithecia. The gene is located on linkage group IV near a putative map location of *acon-3*, a previously characterized conidiation mutant (Springer and Yanofsky, 1989). Comparison of the *acon-3* mutant strain with the NCU07617.4 deletion strain revealed identical phenotypes. Therefore we sequenced the NCU07617.4 allele in the *acon-3* mutant strain. We found a transversion mutation at nucleotide position 820 of the predicted coding sequence. This changes the glutamine at amino acid 273 to a TAG stop codon, thus, prematurely terminating the 692 codon open reading frame. Transformation of the *acon-3* mutant strain with a PCR-generated clone containing 2,118 bp of DNA upstream of the coding region and 419 bp downstream complemented the conidiation defect. Taken together, these results led us to conclude

that the orthologous gene to *medA* in *N. crassa,* NCU07617.4 is the same as the gene mutated in the *acon-3* strain.

Complementation of the *A. nidulans medA* mutants by *acon-3*

Cross-complementation of genes involved in regulating conidiation has been performed for closely related *Aspergillus* and *Penicillium spp*. For example, expression of *A. nidulans abaA* complements defective conidiation and dimorphism of the *P. marneffei abaA* mutant. Moreover, expression of the *P. marneffei stuA* ortholog and the *Penicillium chrysogenum wetA* ortholog complements the defective conidiation of the *A. nidulans stuA* (stunted), and *wetA* mutants, respectively (Borneman et al., 2000; Borneman et al., 2002; Prade and Timberlake, 1994). In contrast, *A. nidulans* and *N. crassa* are much more distantly related.

The *medA15* allele (Clutterbuck, 1969) was generated using nitrous acid. I sequenced the *medA15* allele and found it carries a single transversion (C to A) at nucleotide 295 in the coding region resulting in cysteine codon 81 becoming a stop codon (TGA). I also generated a Δ*medA* strain using the deletion cassette from the FGSC (Program Project Grant GM068087), and homologous replacement of *medA* with *A. fumigatus pyrG* was verified by PCR and Southern blot analysis (Fig. 2.3). Mutation of *medA* in *A. nidulans* caused abnormal conidiophore morphologies including multiple layers of sterigmata and, more rarely, secondary conidiophores. The morphology of conidiophores of Δ*medA* and *medA15* was indistinguishable from each other (Fig. 2.4 b-d). The *medA15* and Δ*medA* mutants produced conidia, however, the conidial yield was

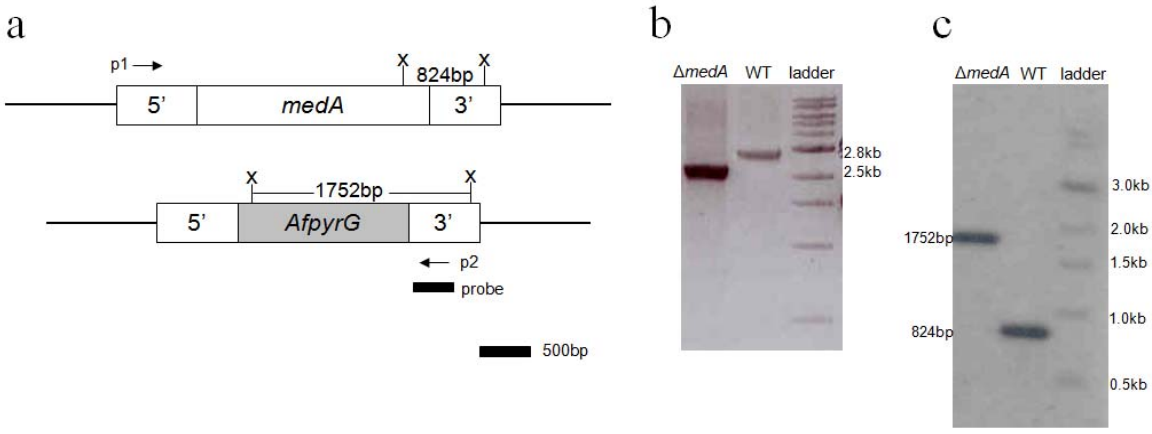

Figure 2.3 Verification of homologous replacement of *medA* with *A. fumigatus pyrG*. (a) Design of the *medA* knock-out cassette from FGSC. The top panel shows a *medA* locus in *A. nidulans* wild type. The bottom panel shows the *medA* coding region replaced by *A. fumigatus pyrG* (*AfpyrG*). Primer sites used to screen Δ*medA* transformants were indicated as p1 (acon3KI-seq1) and p2 (medAKO-ScR). *XhoI* restriction enzyme sites (as 'x') were indicated. (b) PCR-amplified genomic DNA of Δ*medA* and wild type by primers p1 and p2 exhibited the expected size (2.5 kb for Δ*medA*, and 2.8 kb for wild type). (c) Southern blot analysis verified *medA* was replaced by *AfpyrG*. Digested genomic DNA of Δ*medA* and wild type by *XhoI* exhibited the expected size (1,752 bp for Δ*medA*, and 824 bp for wild type).

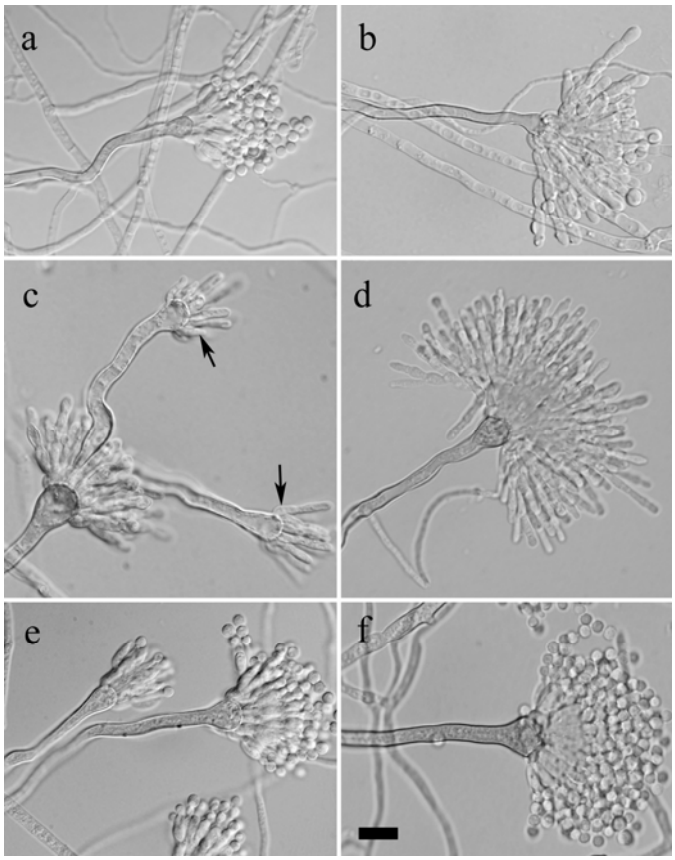

Figure 2.4 Complementation of defective conidiophore morphology and conidial yield in *medA15* and Δ*medA* with *acon-3*.
(a) A conidiophore of *A. nidulans* wild type (A773) produces an ordered progression of cell types from a vesicle to metulae to phialides to conidia. (b-c) Conidiophores of *medA15* (Asum3). (d) A conidiophore of Δ*medA*. Note that both mutants produce abnormally differentiated and reiterated sterigmata and fewer conidia (b and d) compared to wild type (a). Occasionally, both mutants also produce secondary conidiophores from the primary conidiophore (*medA15* shown; arrows in c). (e) Conidiophores of TDC1.23 (*acon-3*(p)::*acon-3*;*medA15*). (f) A conidiophore of RDC21.1 (*acon3*(p)::*acon-3*;Δ*medA*). Expression of *acon-3* via its native promoter restores wild type conidiophore morphology of *medA15* and *ΔmedA*. Scale bar = 10 μm.

less than wild type by ~300-fold and ~90-fold, respectively (Table 2.4). I transformed the medA15 mutant with a construct expression acon-3 by the N. crassa native promoter. Two independent transformants, TDC1.23 and TDC1.29 (*acon-3*(p)::*acon-3*;*medA15*), showed wild-type colonial phenotypes and conidiophore structures when grown on MM at 28 °C (Fig. 2.4e). TDC1.23 and TDC1.29 each contained a single copy of the *acon-3* construct as verified by Southern blot analysis (Fig. 2.5). TDC6.14 and TDC6.19, expressing *medA* via its native promoter (*medA*(p)::*medA*;*medA15*), produced phenotypically normal conidiophores (data not shown). However, this 'promoter region' of 1.6 kb did not contain the full length 5' untranslated region of *medA* (2.1 kb) as determined subsequently by analysis of RNA-seq data. Nonetheless, both of these *medA* and *acon-3* constructs complemented the defective conidiophore morphologies of *medA15*. The defective sexual development of *medA15* was complemented by *medA* expressed by the *A. nidulans* promoter (*medA15*;*medA*(p)::*medA*), but not by *acon-3* expressed by the *N. crassa* promoter (*medA15*;*acon-3*(p)::*acon-3*).

In addition to the *medA15* strain, I expressed *acon-3* in Δ*medA* using the *N. crassa* native promoter (Δ*medA*;*acon-3*(p)::*acon-3*). Since Δ*medA*, and TDC1.23 and TDC1.29 have sexual defects, I first obtained progeny carrying *acon-3* in wild type by crossing TDC1.23 to PW1. The resulting strain RDC20.10 (*acon-3*(p)::*acon-3*;*medA*+) showed normal sexual development, and was crossed to Δ*medA* to mobilize the *acon-3*(p)::*acon-3* construct into Δ*medA*. The resulting strain RDC21.1 (*acon3*(p)::*acon-3*;Δ*medA*) produced morphologically normal conidiophores (Fig. 2.4f) and conidia production was restored to ~74% of the number produced by wild type (Table 2.4).

Table 2.4 Complementation of the *A. nidulans medA15* and Δ*medA* strains

Strain	Genotype	Conidiophore morphology	Conidia production	
			spores/μl	SE*
A773	*medA* wild type	WT	6.54×10^5	8.10×10^4
TN02A25	*medA* wild type	WT	6.03×10^5	7.15×10^4
Asum3	*medA15*	*medA*	2.17×10^3	3.66×10^2
Δ*medA*	Δ*medA*	*medA*	6.60×10^3	1.48×10^3
TDC1.23	*medA15*;*acon-3*	WT	6.18×10^5	1.13×10^5
TDC6.14	*medA15*;*medA*	WT	5.48×10^5	4.56×10^4
RDC21.1	Δ*medA*;*acon-3*	WT	4.48×10^5	3.67×10^4
RDC22.3	Δ*medA*;*medA*	WT	5.06×10^5	4.58×10^4

WT: wild type-like; *medA*: *A. nidulans medA* mutant phenotype (abnormal sterigmata and production of secondary conidiophores). '*': Standard error of triplicates.

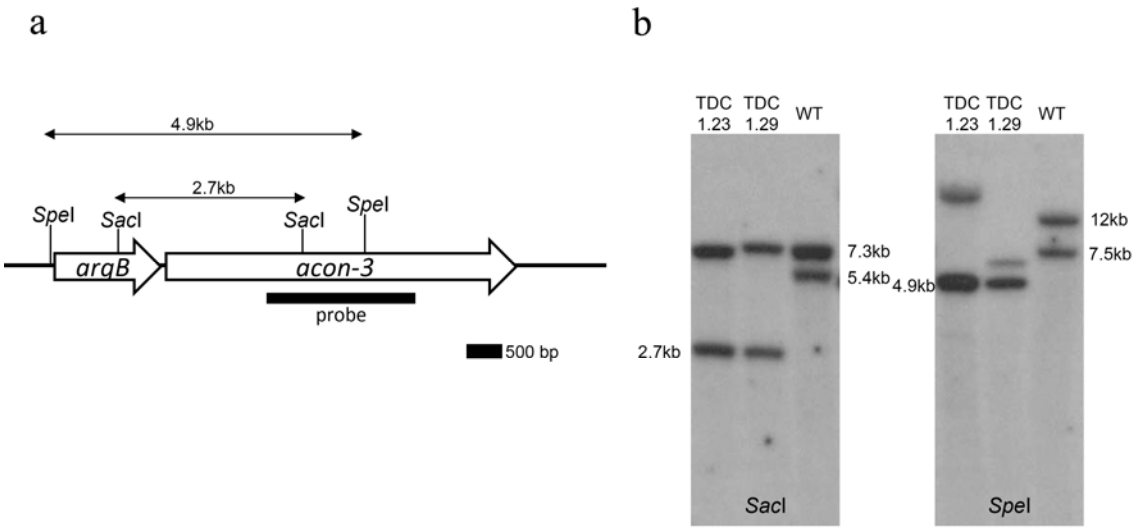

Figure 2.5 Southern blot analysis for a single copy integration of *N. crassa acon-3*.
(a) Design of a *N. crassa acon-3* expressing construct using its native promoter in *medA15*. *Sac*I and *Spe*I restriction enzyme sites were indicated. (b) Southern blot analysis to verify a single copy integration of the *N. crassa acon-3* construct in TDC1.23 and TDC1.29. WT; *N. crassa* wild type 74A-OR23-1VA. Digested genomic DNA of wild type and TDC1.23, and TDC1.29 by *SacI* and *SpeI* exhibited bands of the expected size and number.

Strain RDC22.3 (Δ*medA*;*medA*(p)::*medA*) was obtained by crossing Δ*medA* to TDC6.14, and the progeny were screened by observation of colonial phenotypes and PCR. RDC22.3 produced normal conidiophores (data not shown), and conidia production was equivalent to wild type (Table 2.4). Therefore, the conidiation defects of Δ*medA* and *medA15* were complemented by expression of *acon-3* from the *acon-3* promoter. My attempts to replace the *medA* coding sequence with *acon-3* coding sequence at the *medA* locus failed. Thus, I were unable to assess whether expression of *acon-3* via the large and complex *medA* promoter is sufficient to complement the sexual phenotype. Future work is needed to thoroughly address the *medA* and *acon-3* promoter structures and the role of this structure in expression during sexual and asexual development. Therefore in this report, I have focused on determining whether the MedA proteins have conserved biochemical function for conidiation.

GFP-tagged MedA localized to nuclei

In order to study localization of MedA in *A. nidulans,* I fused it to GFP and integrated the construct replacing native *medA* locus (pDC3.1, and TDC3.38). pDC3.1 was transformed to *A. nidulans* wild type. Replacement of the native *medA* gene with the *medA::eGFP* was verified by Southern blot analysis (Fig. 2.6) and all developmental processes observed were indistinguishable from wild type, indicating that the fusion was functional. Localization of MedA::GFP in TDC3.38 was to nuclei of germlings, vegetative hyphae, and conidiophores (Fig. 2.7). MedA::GFP was also detected in Hülle cells. In dormant ascospores, nuclei were not visible, probably due to being masked by

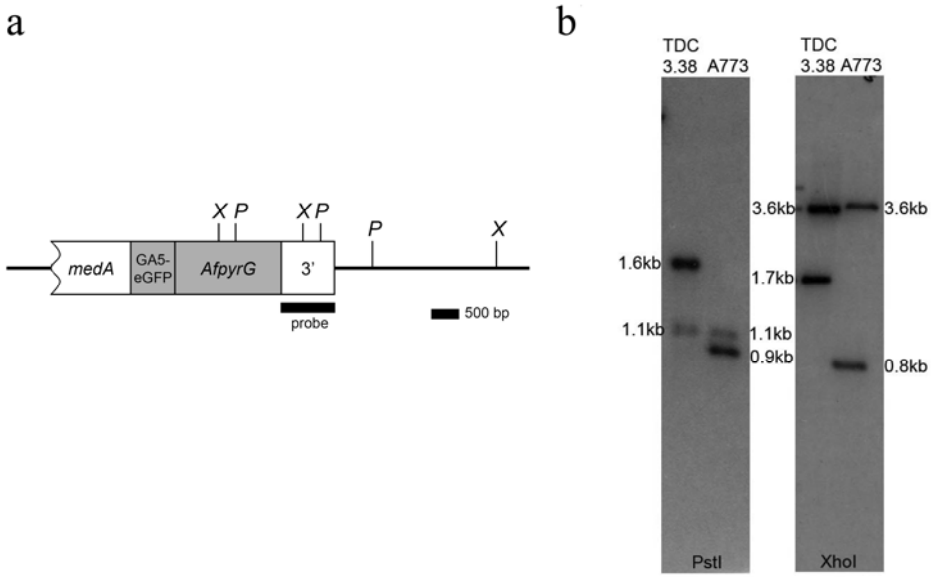

Figure 2.6 eGFP-tagging of *A. nidulans* MedA at the *medA* locus.
(a) Design of a eGFP-tagging *A. nidulans* MedA construct. A 1.3 kb of *medA* coding sequence and a 1 kb of 3' flanking sequence were fused to a pFN03 fragment including *GA5*::*eGFP*::*Aspergillus fumigates* (*AfpyrG*). P: *Pst*I, X: *Xho*I restriction enzyme site, respectively. (b) Southern blot analysis to verify TDC3.38 (*medA*(p)::*medA*::*eGFP*) carried eGFP-tagged MedA at the locus. Digested genomic DNA of wild type (A773) and TDC3.38 by *Pst*I (left panel) and *Xho*I (right panel) exhibited the expected band size to verify homologous recombination of the construct.

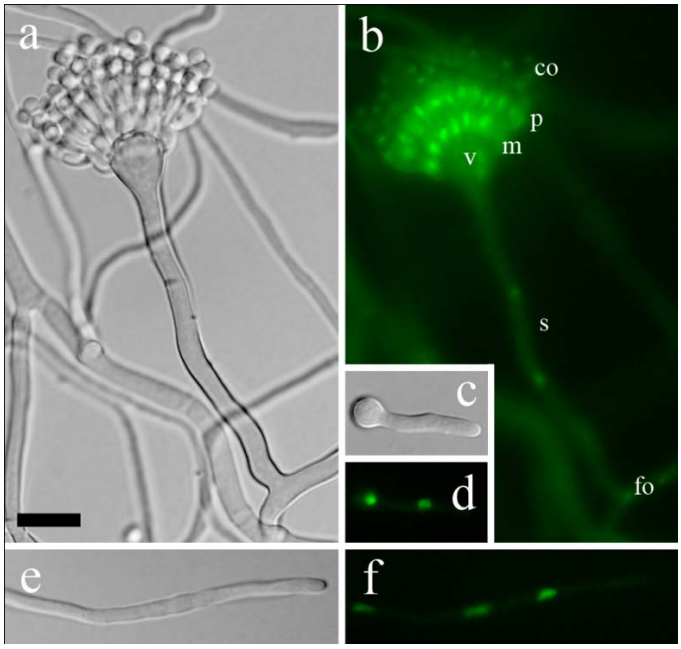

Figure 2.7 MedA::sGFP localization.
A conidiophore is shown (a-b) with a DIC image (a) corresponding to a fluorescence image (b). In all tissue types examined, MedA localizes to nuclei including in foot cell (fo), the stalk (s), vesicle (v), metulae (m), phialides (p), and conidia (co) (b). MedA::sGFP also localizes to nuclei in a germling (d), and vegetative hyphae (f), and their DIC images are shown (c) and (e), respectively. All images are TDC3.38 (*medA*(p)::*medA*::*eGFP*) cultured on MM. Scale bar = 10 μm.

the highly pigmented cell walls, but MedA::GFP was visible in germ tubes immediately after ascospore germination (data not shown).

Previous studies proposed that the MedA proteins act as transcriptional regulators (Lau and Hamer, 1998; Ohara et al., 2004). In addition, GFP-tagged MedA proteins localized to nuclei both in *F. oxysporum* (Ohara et al., 2004), and in *A. nidulans* in this study, which supports a role for MedA as a transcriptional regulator. However, no known nuclear localization signal, DNA binding domains, or protein interaction domains were recognized in our analysis of the *A. nidulans* sequence.

Expression analysis

It has been reported that *A. nidulans medA* produces two transcripts that are expressed immediately after induction of conidiation (Busby et al., 1996). Since expression level may be critical for the ability to complement the conidiation defect, I examined the expression levels of *medA* in *A. nidulans*, and *acon-3* in the *A. nidulans medA15* transformants. In the *A. nidulans* wild type strain (A4), *medA* transcripts were present through all stages of conidiation examined by northern blot analysis. I observed constitutive expression of the previously reported 4.2 kb transcript. A less distinct smaller band, possibly corresponding to 3.8 kb was observed only at some of the time points (Fig. 2.8a).

Remarkably, *acon-3* expressed from its native promoter was developmentally regulated in the *medA15* mutant (TDC1.23). Expression was observed only very late in development at the 16 and 24 h time points (Fig. 2.8b). In *N. crassa*, *acon-3* expression

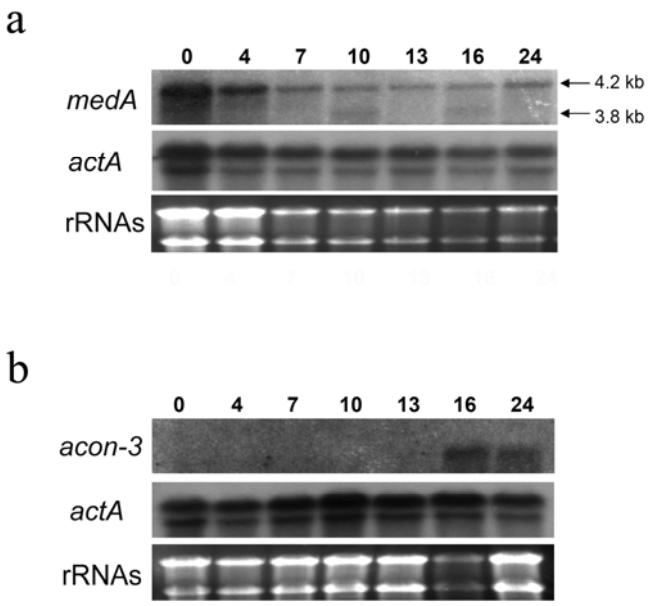

Figure 2.8 Northern blot analysis.
(a) Expression of *medA* in *A. nidulans* wild type (A4) at 0, 4, 7, 10, 13, 16, and 24 h. RNA quality was verified with an *A. nidulans actA* probe. *medA* was constitutively expressed through all developmental stages. Two putative transcripts (4.2 kb and 3.8 kb) of *medA* were detected. (b) Expression of *acon-3* in the *A. nidulans* TDC1.23 (*acon-3*(p)::*acon-3*;*medA15* strain). *acon-3* expression was detected only at 16 and 24 h.

was detected at 10 h post-induction, peaking at 18 h, and then declining (From our Illumina RNA-seq data, not shown). Thus, I hypothesize that the low level of *acon-3* expression during early stages of conidiation in *A. nidulans*, not detected in this northern blot, is sufficient to complement the *medA15* mutant. I do not know the basis for the increased expression detected at 16 and 24 h, however, one possibility is that *acon-3* auto-regulates its expression at these later time points. Previously, Busby and colleagues demonstrated that *brlA* was induced prematurely in the *medA* mutant compared to wild type, suggesting that MedA acted to repress *brlA* early in development. They also found that an extra ectopic copy of *brlA* suppressed the *medA15* conidiation defect (Busby et al., 1996). I performed northern blot analysis to test the possibility that *brlA* expression may be elevated by expressing *acon-3* in the *medA15* strain. The *brlA* locus has two overlapping transcripts, termed *brlAα* and *brlAβ*, and *brlAβ* transcription initiates about 1 kb upstream of the *brlAα* transcription start site (Han et al., 1993; Prade and Timberlake, 1993). Both were detected in wild type at 10 h post-induction, whereas the *brlA* transcripts in *medA15* (Asum3) were first detected at 4 h post-induction (Fig. 2.9). This result agreed with the previous findings that the *medA* mutation led to premature expression of *brlA* in *A. nidulans.* Busby *et al*. observed that expression of *brlA* in wild type was detected when the cells were differentiating vesicles and metulae (at approximately 7-10 hours in our study) (Busby et al., 1996). In TDC1.23 (*acon-3*(p)::*acon-3*;*medA15*) *brlA* expression was first detected at 7 h post induction at a low level. These results indicate that expression of *acon-3* partially restored the wild type

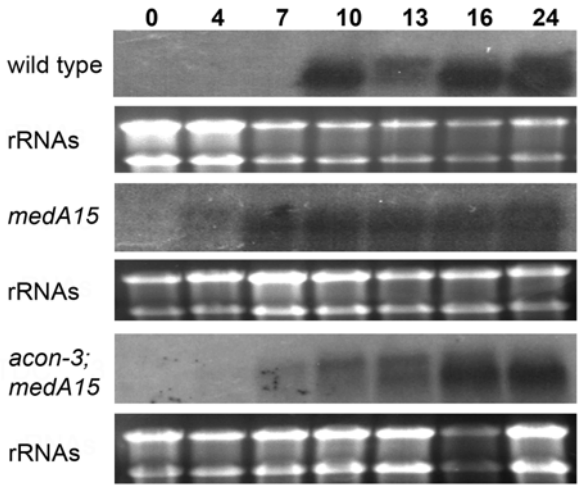

Figure 2.9 Expression of *brlA*.
Expression of *A. nidulans brlA* in *A. nidulans* wild type (A4), *medA15* (Asum3), and TDC1.23 (*acon-3(p)*::*acon-3*;*medA15*) at 0, 4, 7, 10, 13, 16, and 24 h. In wild type, *brlA* expression was first detected at 10 h, whereas at 4h in *medA15*. In TDC1.23 (the *acon-3(p)*::*acon-3*;*medA15*), *brlA* expression was first detected at 7 h, which showed a restored temporal pattern of *brlA* expression similar to wild type.

temporal pattern of *brlA* expression (Fig. 2.9) despite the lack of detectable expression of *acon-3* at that time by northern blot analysis (Fig. 2.8b).

CONCLUSION

Disruption of *medA* orthologs across many ascomycete species results in altered asexual development. However, the role of MedA is enigmatic. For example, the phenotypic consequences in asexual development of *A. nidulans* are reiterated conidiation cell types, while in *N. crassa* the mutation blocks major constriction chain formation, and in *M. oryzae* the mutation results in reorganization of conidiogenous cell pattern. Therefore, it is reasonable to expect that, given morphological differences among these species, the biochemical activity of MedA proteins may have specialized for these different species. However, our data demonstrate that biochemical activity is retained in two species that diverged at least 215 million years ago.

I am interested in how pathways governing asexual sporulation have evolved. Our results support the hypothesis that the last common ancestor of the ascomycetes possessed a gene with activity similar to the extant *medA* orthologs in *A. nidulans* and *N. crassa*. I now recognize at least three patterns for the evolution of genes that regulate conidiation in ascomycetes: (i) *brlA* and *fl* are non-homologous genes with analogous roles as the key regulators; (ii) *flbD* and *rca-1* are orthologs with retained biochemical function that lack an analogous role in conidiation; (iii) *medA* and *acon-3* are orthologs with both a conserved biochemical function and a role in conidiation. Therefore, any model to explain the evolution of regulatory pathways governing conidiation should

consider these three patterns. Current data cannot distinguish whether a conidiation pathway existed in the common ancestor and diverged to the extant forms by recruiting novel regulators (*brlA*, *fl*), or whether the pathways evolved from a common tool kit of genes that are particularly suited to regulate development with the addition of unique regulators (*brlA*, *fl*). Making this distinction will require extending these analyses to additional species across relevant phylogenetic scales.

CHAPTER III

EVOLUTION OF TWO TRANSCRIPTION FACTORS, AbaA AND StuA IN *Aspergillus nidulans* AND *Neurospora crassa*

SUMMARY

Aspergillus nidulans StuA and *Neurospora crassa* ASM-1 are orthologous APSES (ASM-1, PHD1, SOK2, Efg1, and StuA) transcription factors that are highly conserved across a diverse group of fungi. StuA and ASM-1 have a role in asexual (conidiation) and sexual development in both *A. nidulans* and *N. crassa*. AbaA is an ATTS/TEA transcription factor, and disruption of AbaA results in disrupted conidiation and an abnormal conidiophore structure in *A. nidulans*. The ATTS/TEA transcription factor has not been characterized in *N. crassa*. To understand how these transcriptional regulators evolved for coordinating conidiation in ascomycetes, I examined conservation of function of StuA and AbaA and the *N. crassa* orthologs in conidiation. StuA and ASM-1 share 47% identity and 58% similarity in their amino acid sequences. Expression of a single copy of *asm-1* using the *N. crassa* native promoter complemented defective conidiophore morphology and conidia yield in *stuA1*, which suggested that biochemical function of StuA and ASM-1 was conserved. *Neurospora crassa* AbaA (NCU02612, designated NcAbaA) has a weak sequence similarity to *A. nidulans* AbaA (28% identity for 97 amino acids), but microsynteny supports orthology between these proteins. Expression of *NcabaA* using *N. crassa* native promoter, *alcA* promoter, and *A. nidulans abaA* promoter did not complement defective conidiation in *abaA14*. This suggested that

unlike StuA and ASM-1, AbaA and NcAbaA do not share either biochemical function or association with conserved downstream factors. Thus, these two pairs of orthologs have distinct evolutionary patterns: (i) orthologs with similar biochemical function and an analogous role in conidiation (StuA and ASM-1), and (ii) orthologs with biochemical function not conserved and roles in conidiation (AbaA and NcAbaA)

INTRODUCTION

Genes that regulate conidiation are well characterized in *A. nidulans*. *brlA* is a key regulatory gene, and *brlA* → *abaA* → *wetA* constitute a central genetic pathway of conidiation (Clutterbuck, 1969; Timberlake and Marshall, 1988). A group of regulators called 'fluffy low *brlA*' genes including *flbC* and *flbD* induce expression of *brlA*, and disruption of these genes causes production of undifferentiated masses of vegetative hyphal growth and delayed conidiation (Wieser et al., 1994). Another group of conidiation regulators include the developmental modifiers, StuA and MedA. Loss of function mutants of *stuA* and *medA* alter the spatial and temporal expression of the key regulator of *brlA*. The *stuA* and *medA* mutants produce abnormally differentiated conidiophore structures, but are able to produce conidia (Clutterbuck, 1969; Miller et al., 1992). Many of these characterized conidiation regulators are transcription factors (TFs) including, BrlA and FlbC (C_2H_2 zinc finger), FlbD (Myb-like), AbaA (ATTS/TEA), and StuA (APSES) (Adams et al., 1990; Andrianopoulos and Timberlake, 1994; Wieser and Adams, 1995; Wieser et al., 1994; Wu and Miller, 1997).

StuA is an APSES (ASM-1, PHD1, SOK2, Efg1, and StuA) transcription factor. The *A. nidulans stuA1* mutant exhibits normal vegetative hyphal growth, but produces shortened aerial stalks on conidiophores and fewer conidia compared to wild type (Clutterbuck, 1969; Miller et al., 1992). In addition, StuA is required for correct spatial expression of *brlA* and *abaA*. By *in situ* localization using galactosidase, BrlA:β-Gal localizes in vesicles, sterigmata, and immature conidia in wild type. In contrast, BrlA:β-Gal localizes in all tissue types of conidiophores and vegetative hyphae in the *stuA1* mutant (Aguirre, 1993). In addition, AbaA:β-Gal localizes in metulae, phailides, and immature conidia in wild type. However, AbaA:β-Gal localizes to vegetative hyphae, aerial stalks, vesicles, and sterigmata in the *stuA1* mutant (Miller et al., 1992).

StuA proteins are well characterized in various fungal species including *A. nidulans*, *A. fumigatus*, *Saccharomyces cerevisiae*, *Candida albicans*, *Neurospora crassa*, *Fusarium oxysporum*, *Penicillium marneffei*, and *Ustilago maydis*. In *S. cerevesiae*, there are two characterized proteins homologous to StuA, PHD1 and SOK2. Over-expression of PHD1 and deletion of SOK2 enhances pseudohyphal growth, which suggests that PHD1 and SOK2 have distinct roles as an activator and a repressor for pseudohyphal formation, respectively (Gimeno and Fink, 1994; Ward et al., 1995). In *C. albicans* and *U. maydis*, the StuA homologous proteins, Efg1p and Ust1 play a role in the dimorphic switch. For example, over-expression of *EFG1* induced filamentous growth in *C. albicans* and deletion of *ust1* leads to filamentous growth in budding haploid *U. maydis* wild type (Garcia-Pedrajas et al.; Stoldt et al., 1997). In *N. crassa*, *P. marneffei*, *F. oxysporum*, and *A. fumigatus*, disruption of *stuA* orthologs results in

abnormal conidiophore morphology including shortened aerial cells (Aramayo et al., 1996; Borneman et al., 2002; Ohara and Tsuge, 2004; Sheppard et al., 2005). Together, these previous studies indicate that StuA protein homologs are associated with fungal morphogenesis and development.

AbaA is an ATTS/TEA transcription factor. A loss of function mutant of *abaA* produced abnormal conidiophores with 'abacus'-like sterigmata and no conidia formation in *A. nidulans* (Andrianopoulos and Timberlake, 1994; Clutterbuck, 1969). The ATTS/TEA DNA-binding motif is present in the human transcription enhancer TEF-1, and in the *Drosophila melanogaster* scalloped gene required for differentiation of nervous system. In fungi, *S. cerevisiae* transcription factor TEC1 contains the ATTS/TEA DNA-binding motif, and TEC1 is associated with the mitogen-activated protein kinase pathway that regulates invasive growth (Campbell et al., 1992; Laloux et al., 1990; Wang et al., 2009; Xiao et al., 1991) . In *P. marneffei*, Δ*abaA* is aconidial and produces reiterating cells that do not produce phialides. In addition, Δ*abaA* fails to switch from filamentous growth to yeast growth which suggests that AbaA plays a role in the dimorphic switch in *P. marneffei* (Borneman et al., 2000).

Expression of *P. marneffei stuA* using either its native *P. marneffei* promoter or *A. nidulans stuA* promoter complements defective conidiation in *A. nidulans* Δ*stuA* (Borneman et al., 2002). Moreover, expression of *A. nidulans abaA* using its native *A. nidulans* promoter complements defective conidiation and the dimorphic switch in *P. marneffei* Δ*abaA* (Borneman et al., 2000). These results suggested that these two conidiation regulators retain biochemical function for conidiation between *A. nidulans*

and *P. marneffei*, two members of the family Trichocomaceae. Prior to this dissertation, no studies examined the conservation of these two transcriptional regulators in distantly related fungal species.

I assessed functional conservation of the two conidiation regulators, StuA and AbaA in two distantly related ascomycetes, *A. nidulans* and *N. crassa* using a cross-species complementation approach. Expression of the *N. crassa stuA* ortholog, *asm-1* using its native *N. crassa* promoter complemented defective conidiation in *A. nidulans stuA1*, which suggests that orthologous StuA proteins have a conserved biochemical function between *A. nidulans* and *N. crassa*. In contrast, expression of *N. crassa abaA* ortholog (*NcabaA*) using its native *N. crassa* promoter, the alcohol-inducible (*alcA*) promoter, and the *A. nidulans abaA* promoter did not complement defective conidiation in *abaA14*. This suggests that an important function of AbaA proteins is not conserved between two species. Thus, given distinct evolutionary patterns for these two regulators, the evidence of a single genetic machinery for conidiation maintained across all ascomycetes is not supported.

MATERIALS AND METHODS

Strains, media, and growth conditions

A list of *A. nidulans* strains, and plasmids and constructs used in this study is shown in Table 3.1 and Table 3.2, respectively. Minimal medium (MM) and complete medium (CM) for growing *A. nidulans* strains were prepared with appropriate supplements as described (http://www.fgsc.net). To induce gene expression using the

Table 3.1 Strains used in this study (*abaA* and *stuA*)

Strains	Genotype	Source
A4	*A. nidulans* wild type	FGSC*
A585	*biA1*; *stuA1*; *veA1*	FGSC*
A590	*biA1*; *abaA14*; *veA1*	FGSC*
A773	*pyrG89*; *pyroA4*;*wA3*; *veA1*	FGSC*
PW1	*biA1*; *argB2*; *methG1*;*veA1*	Goc et al. (1987)
Asuku22	*pyrG89*; *argB2*; *pyroA4*; *nkuA*::*argB*; *riboB2*; *veA1*	This study
ASL91	*pyrG89*; *argB2*; *wA3*; *veA1*	This study
Asus5	*biA1*; *stuA1*; *argB2*; *pyrG89*; *wA3*; *veA1*	This study
Δ*stuA*	*ΔstuA*; *N. crassa pyr-4*; *pyrG89*; *pyroA4*; *veA1*	This study
RDC-abaA14	*abaA14*; *argB2*; *pyrG89*; *wA3*; *veA1*	This study
TDC7.21	*stuA1*; *argB2*; *pyrG89*; *wA3*; *veA1*; *asm-1*::*argB*	This study
TDC8.1	*abaA::GA5::eGFP::A. fumigatus pyrG; pyrG89;pyroA4*	This study
TDC9.2/9.5	*alcA*(p)::*asm-1*; *stuA1*; *argB*; *argB2*; *pyrG89*	This study
TDC10.5/10.17	*NcabaA*(p):: *NcabaA*; *abaA14*; *argB*; *argB2*; *pyrG89*	This study
TDC11.1/11.3	*alcA*(p)::*NcabaA*; *argB*; *argB2*; *pyrG89*; *abaA14*	This study
TDC12.1/12.2	*A. nidulans abaA*(p)::*NcabaA*; *argB*; *abaA14*; *argB2*; *pyrG89*	This study

* Fungal Genetics Stock Center (McCluskey, 2003)

Table 3.2 Plasmid/construct used in this study (*abaA* and *stuA*)

Plasmids	Characteristics	Source
pGEM-T Easy	TA cloning vector	Promega
pENTR-TOPO	Entry vector for GATEWAY cloning	Invitrogen
pSL-GFP	*alcA*::*ccdB*::*sGFP*; *pyr-4*; *bla*	Lee and Shaw, 2008
pMT-ove	*alcA*::*ccdB*; *argB*;*bla*	Toews *et al*., 2004
pFN03	*GA5*::*eGFP*; *A. fumigatus pyrG* (*AfpyrG*)	Yang *et al*., 2004
pDC3.1	*asm-1* coding region with 1.5 kb up- and 1.1 kb downstream sequence; *argB*	This study
pDC4.1	*asm-1* coding sequence cloned to pENTR-TOPO	This study
pDC5.1	*alcA*(p)::*asm-1*; *argB*	This study
pDC6.1	*asm-1* coding sequence without stop codon cloned to pENTR-TOPO	This study
pDC7.1	*alcA*(p)::*stuA*::*sGFP*; *N. crassa pyr-4*	This study
pGEM-stuAKO	Δ*stuA*; *N. crassa pyr-4* construct into pGEM-T Easy vector	This study
pDC8.1	*abaA*(p)::*abaA*; *argB*	This study
PDC9.1	*NcabaA*(p)::*NcabaA*; *argB*	This study
pDC10.1	*NcabaA* coding sequence cloned to pENTR-TOPO	This study
pDC11.1	*alcA*(p)::*NcabaA*; *argB*	This study
pDC12.1	*abaA*(p)::*NcabaA; N. crassa pyr-4*	This study

alcA promoter (Felenbok et al., 2001), conidia were incubated in either liquid or solid MM without glucose containing 0.5% ethanol and 1% glycerol. Fungal strains were cultured at 28 °C unless otherwise indicated. All reagents for media, supplements, and buffers used were purchased from Sigma (St. Louis, MO) unless otherwise indicated. All plasmids were stored and amplified in *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA). Conidia production studies were carried out in triplicate on MM agar with appropriate supplements as described previously (Chung et al., 2011).

Identification of orthologs

We found the putative orthologs of *A. nidulans stuA* and *abaA* in *N. crassa* genome using the BLASTP program as previously described (Chung et al., 2011). Briefly, to find the putative ortholog of *A. nidulans* StuA, we used a 621 amino acid sequence of *A. nidulans* StuA (ANID_05836) as a query sequence against the *N. crassa* genome. Then,the amino acid sequence of the putative *N. crassa* ortholog (NCU01414) was used as a query sequence against *Aspergillu*s genome to confirm their orthology by a reciprocal BLAST search. Sequence alignment was performed and visualized with GeneDoc software (Nicholas et al., 1997).

To find the putative ortholog of *A. nidulans* AbaA, we used a 796 amino acid sequence of *A. nidulans* AbaA (ANID_00422) as a query sequence against the *N. crassa* genome. Then, the amino acid sequence of the candidate *N. crassa* ortholog (NCU02612; designated NcAbaA) was used as a query sequence against *A. nidulans*

genome for reciprocal BLAST to confirm their orthology. Amino acids sequences of adjacent genes to *abaA* and *NcabaA* (ANID_00421 and NCU02611, respectively) were compared using bi-directional BLAST search to evaluate microsynteny.

Molecular cloning procedures

Plasmids to express *N. crassa* orthologs in the *A. nidulans* conidiation mutants were created as previously described (Borneman et al., 2000; Borneman et al., 2002; Chung et al., 2011). Briefly, pDC3.1 to express *asm-1* by the native *N. crassa* promoter was designed. A list of oligonucleotides used in this study was shown in Table 3.3. A DNA fragment of 4,566 bp including the 1,932 bp of coding region, 1,516 bp of upstream sequence and 1,118 bp of downstream sequence of *asm-1* was amplified using the primers NcstuAF and NcstuAR. The PCR product was digested using *Xba*I, and ligated into the *Xba*I-digested pTA-argB vector, that carried the *A. nidulans argB* sequence as a selectable marker.

Table 3.3 Oligonucleotides used in this study (*abaA* and *stuA*)

Designation	Sequence (5'→ 3')
NcstuAF	TGTGAATTCTAGAAACCGAGAGACGAAGGCT
NcstuAR	CCTTGCTTTCTAGAGCCCCCGAGGTATTCC
NcstuA-oveF	CACCATGAACCCCAACACACCAGC
NcstuA-oveR	TTATCTCCGCCTGTGGGCCG
stuAKO5F	GTGCCTGTACCTTTGCCTGTGCCC
stuAKO5R	TCTCGATGCAGTGCCCTGTGTAGTAGCTCTCGGTGACT
stuAKO3F	TAAGGCACACGGGCAAGGATTGCGAAAGAGCTCATAT
stuAKO3R	TGATCGGAACTTGCGCGAGGCCAT
Ncpyr4F	ACAGGGCACTGCATCGAGAGTTC
Ncpyr4R	TCCTTGCCCGTGTGCCTTACAAAT
stuAKOneF	CAACGACCTGCTACGACCTGTTAC
stuAKOneR	CGGAGACCTAGCATAGTGAGCAAC
stuAGFP-TOPOF	CACCATGGCCAGCATGAATCAACCTCA
stuAGFP-TOPOR	ACGACGAGCACTTATCAGACTGCC
AbaAGFP1a	CGAAGACGGGAGGCGCACGATGGAGG
AbaAGFP2a	CAGCGCCTGCACCAGCTCCGACAGCCTCAACCGCAGTAT
AbaAGFP3a	AGTGCCTCCTCTCAGACAGTAGCCTCCTTTACCATGTC
AbaAGFP4a	CGGTGGTGCTCCTTCCGCCTGTTTCA
pFN03F	GGAGCTGGTGCAGGCGCTGGAG
pFN03R	CTGTCTGAGAGGAGGCACTGAT
AbaAGFPNeFa	GGTGGGCAAAAACCTTCACGAAAC
AbaAGFPNeRa	TATGGGTGGTGCTCGATTTGCAGC
NcstuASF	CGACACAACTGGCCAAGTAGCA
NcAbaAwalkF1	GCTCTGGGGTCGGAACATGTTA
NcAbaAwalkR1	GTGAAGCAACAACAGAAGTCTG
NcstuASR	CCTGGCCTGCGTACATCTCATT
AnAbaAcomF	TTCTCCACAACTAGTCAATCTTTGA
AnAbaAcomR	ATGAGAGAACTAGTATACGCCTCG
AbaA-Ch1	TCGATAGGTGCAAATCCAGGTCG
AbaA-Ch2	GGA GCA GAC CCC AAG ATT CGC TC
AbaA-Ch3	GAATCTTGGGGTCTGCTCCATGGAGTTGCCAGTCAGAC
NcAbaAR-xbaI	AGCCCGTACTCTAGACTAATCAAGC
NcAbaACh-Fxba	CACACGTTCTAGACAGTACCT
NcAbaACh-Rxba	TTTATCGTCTAGAGACCTACTACT

pDC5.1 was designed to over-express *asm-1* in *stuA1* using the *alcA* promoter as previously described using the GATEWAY cloning vector (Toews et al., 2004; Upadhyay and Shaw, 2006). A DNA fragment of 1,936 bp including the *asm-1* coding sequence was amplified using the primers NcstuA-oveF and NcstuA-oveR and cloned into pENTR-TOPO (Invitrogen) to generate pDC4.1. Plasmid DNA of pDC4.1 was isolated and used for the reaction with GATEWAY designation vector, pMT-ove, which resulted in pDC5.1.

Strain A585 was crossed to strain ASL91 to generate strain Asus5 (*argB2*; *pyrG89*; *stuA1*). The plasmids, pDC3.1, and pDC5.1 were transformed into Asus5 using a protoplast transformation method (Yelton et al., 1984). This resulted in generation of a pDC3.1 transformant TDC7.21 (*asm-1*(p)::*asm-1*; *stuA1*), and pDC5.1 transformants TDC8.1 and TDC8.5 (*alcA*(p)::*asm-1*; *stuA1*). A single copy insertion of the plasmid pDC3.1 in Asus5 was verified by PCR and Southern blot analysis.

To generate Δ*stuA*, the knock-out cassette was generated using fusion PCR method (Yu et al., 2004). A 1,065 bp of upstream sequence and a 1,085 bp downstream sequences of *A. nidulans stuA* were amplified using the primers stuAKO5F and stuAKO5R, and stuAKO3F and stuAKO3R, respectively. A 1,880 bp DNA fragment of *N. crassa pyr-4* was amplified using the primers Ncpyr4F and Ncpyr4R. The final fusion PCR product of 3,829 bp was generated using the primers stuAKOneF and stuAKOneR. This cassette was cloned to pGEM-T Easy vector (designated pGEM-stuAKO), and plasmid of pGEM-stuAKO was transformed into *A. nidulans* wild type Asuku22 using the protoplast transformation method to generate Δ*stuA*. PCR and Southern blot analysis

were performed to verify homologous recombination of the *stuA* knock-out construct at the native *stuA* locus using *N. crassa pyr-4* as a selectable marker.

To express *A. nidulans abaA* using its native *A. nidulans* promoter, a DNA fragment of 4,653 bp including 2,492 bp of the *A. nidulans abaA* coding sequence, and 1,575 bp of upstream and 585 bp of downstream of *abaA* was amplified using the primers AnabaAcomF and AnabaAcomR. The PCR product was *SpeI*-digested and ligated into *SpeI*-digested pTA-argB to generate pDC8.1. To express *NcabaA* using the native promoter, a DNA fragment of 4,712 bp including 3,528 bp of the *NcabaA* coding sequence, 662 bp of upstream and 522 bp of downstream of *NcabaA* was amplified using the primers NcabaAF-xbaI and NcabaAR-xbaI. Then the PCR product was *XbaI*-digested and ligated to *XbaI*-digested pTA-argB to generate pDC9.1. Though my original plan was to include ~1.5 kb of upstream sequence, as I did for the *asm-1* expression construct (pDC3.1), here I included only a 662 bp of upstream sequence for the *NcabaA* expression construct (pDC9.1) to avoid overlap with the adjacent gene (NCU02611).

To over-express *NcabaA* using the *alcA* promoter, a DNA fragment of 3,532 bp including the *NcabaA* coding sequence was amplified using the primers AnabaA-oveF and AnabaA-oveR, and cloned into pENTR-TOPO (Invitrogen) to generate pDC10.1. Plasmid DNA of pDC10.1 was isolated and used for reaction with GATEWAY designation vector, pMT-ove (Toews et al., 2004) to generate pDC11.1. To express *NcabaA* using *A. nidulans abaA* promoter, a DNA fragment of 5,285 bp including 901 bp of upstream sequence of the *abaA* start codon, 3,861 bp of the *NcabaA* coding

sequence and 523 bp of downstream sequence of *NcabaA* were amplified using the primers AbaA-ch1 and AbaA-ch2, and AbaA-ch3 and NcAbaAR-xbaI, respectively. The final fusion PCR product of 5,069 bp was amplified using the primers NcAbaCh-Fxba and NcAbaCh-Rxba, *XbaI*-disgested, and cloned into *XbaI*-digested pTA-argB to generated pDC12.1. Strain A590 was crossed to strain ASL91 to generate strain RDC-abaA14 (*argB2*; *pyrG89*; *aba14*). Plasmids of pDC8.1, pDC9.1, pDC11.1 and pDC12.1 were transformed into RDC-abaA14, respectively to evaluate whether the construct complemented defective conidiation in *abaA14*.

GFP tagging of *A. nidulans* StuA and AbaA

Aspergillus nidulans StuA protein was tagged by GFP (green fluorescence protein) using the GATEWAY cloning system (Invitrogen) as previously described (Toews et al., 2004; Upadhyay and Shaw, 2006). Briefly, I amplified a DNA fragment of 2,008 bp including *stuA* coding sequence using the primers stuAGFP-TOPOF and stuAGFP-TOPOR, and the amplicon was cloned to pENTR-TOPO to generate pDC6.1. Then *stuA* gene was transferred from pDC6.1 to a destination vector, pSL-sGFP to generate pDC7.1. Then, pDC7.1 was transformed into the *A. nidulans* wild type strain, A773, and GFP signals were observed to screen the transformants using a microscope.

Aspergillus nidulans AbaA was tagged by GFP at the native *abaA* locus using fusion PCR protocol as previously described (Chung et al., 2011). Briefly, a DNA fragment of 1,177 bp including the *abaA* coding sequence and a 1,413 bp downstream sequence were amplified by the primers AbaAGFP1a and AbaAGFP2a, and

AbaAGFP3a and AbaAGFP4a, respectively. A 2,644 bp of the GA(Gly-Ala)5-eGFP-*A. fumigatus pyrG* fragment was amplified from plasmid, pFN03 (Yang et al., 2004) using the primers pFN03F and pFN03R. The final PCR product of 5,024 bp was fused from the three fragments by the primers AbaGFPNeFa and AbaGFPNeRa. The final PCR product was transformed into *A. nidulans* wild type Asuku22, and PCR and Southern blot analysis were performed to verify GFP-tagging of AbaA at the native locus.

Southern blot analysis

Southern blot analyses were conducted following standard procedures (Sambrook et al., 1989). To verify a single copy insertion of the *N. crassa asm-1* expression construct (pDC3.1) in *stuA1*, genomic DNA of TDC7.21 (*asm-1*(p)::*asm-1*; *stuA1*) and wild type (A4) were isolated from mycelia and digested by *StuI* and *XhoI*. The probe was a 1,367 bp PCR fragment amplified using the primers NcstuASF and NcstuASR. To verify deletion of *stuA*, genomic DNA of Δ*stuA* and wild type (A4) were extracted from mycelia and digested by *HindIII* and *XhoI*. The probe was a 1,085 bp PCR fragment amplified using the primers StuAKO3F and StuAKO3R. To verify a single copy insertion of the *NcabaA* expression construct (pDC9.1), genomic DNA of TDC10.5 and TDC10.17 (*NcabaA*(p)::*NcabaA*; *abaA14*) and wild type (A4) were extracted from mycelia and digested by *EcoRI* and *XhoI*, respectively. The probe was a 2,650 bp PCR fragment amplified using the primers NcAbaA-walkF1 and NcAbaA-walkR1. To verify integration of the *abaA::eGFP* construct at the *abaA* locus, genomic DNA of TDC8.1 and wild type were isolated from mycelia and digested by *EcoRI* and

HindIII. The probe was a 1,413 bp PCR fragment amplified using the primers AbaAGFP3a and AbaAGFP4a.

Light microscopy

Germlings or hyphae were grown on coverslips in liquid MM medium with appropriate supplements, and cell morphology and GFP signals of the GFP fusion proteins were observed under the microscope as previously described (Shaw and Upadhyay, 2005). Localization of StuA::GFP and AbaA::GFP in live cells were performed as previously described (Riddell, 1950; Upadhyay and Shaw, 2006).

RESULTS

Putative orthologous genes of *A. nidulans stuA* and *abaA* are present in *N. crassa*

We used BLASTP to identify putative *N. crassa* orthologs for *A. nidulans* StuA and AbaA. The amino acid sequences of StuA and AbaA (ANID_05836 and ANID_00422) were used to query the *N. crassa* genome. The results showed that NCU01414, previously characterized as *asm-1* (Aramayo et al., 1996), was the ortholog of *stuA* (E-value 0), and ASM-1 and StuA shared 60% sequence similarity and 48% identity. Moreover, both StuA and Asm-1 sequences contain APSES DNA-binding domain (Aramayo et al., 1996; Dutton et al., 1997) at 129-235 and 116-222 amino acid residues, respectively (Fig. 3.1). The orthology was confirmed by best-hit in a bi-directional comparison between StuA and ASM-1.

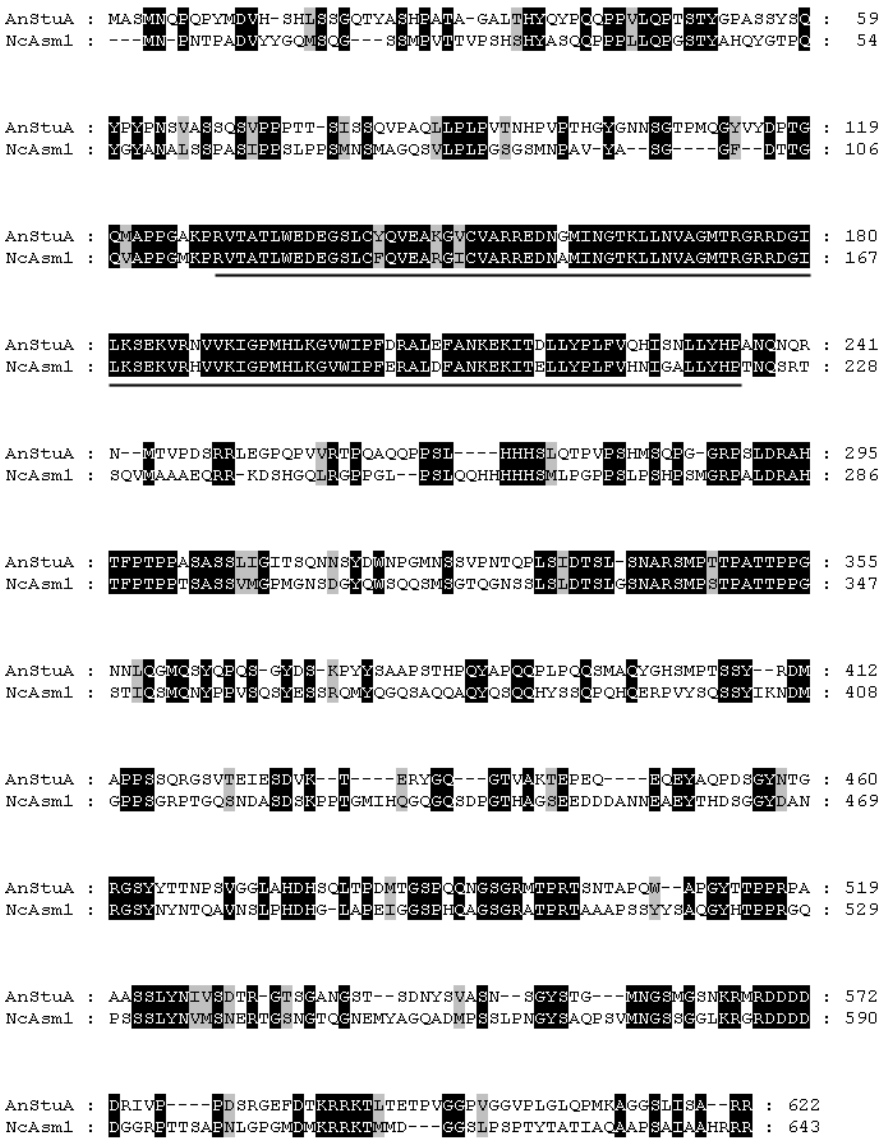

Figure 3.1 Sequence similarity between *A. nidulans* StuA and *N. crassa* ASM-1.
The amino acid sequence of *A. nidulans* StuA (AnStuA) and *N. crassa* ASM-1 (NcAsm1) were aligned using GeneDoc. Similar and identical residues are indicated in gray and black, respectively. StuA and ASM-1 are highly conserved (48% identity and 60% similarity). APSES DNA-binding regions of the StuA sequence (amino acid residues 129-325) and the ASM-1 sequence (amino acid residues 116-222) are indicated by underlines below the sequence.

Unlike StuA, AbaA has not been characterized functionally across many fungal species. We found putative orthologous AbaA proteins in *Aspergillus* and *Penicillium* species (E-value 0), *Sclerotinia sclerotiorum* (31% identity in 471 amino acids), and *Ustilago maydis* (28% identity in 383 amino acids) using the 796 amino acid sequence of *A. nidulans* AbaA as a query. In *N. crassa*, NCU02612 (designated NcAbaA in this study) was identified as the putative ortholog of AbaA (E-value 10e-04), however, AbaA and NcAbaA showed very weak sequence similarity with 34% identity in a 90 amino acid region from 147 to 236 of NcAbaA. Furthermore, queries of the *A. nidulans* genome using NCU02612 did not identify any hits when using the full sequence of the AbaA protein. We next compared the adjacent genes (ANID_00421 and NCU02611), and found that the orientation of the open reading frames (ORFs) and relative locations to AbaA and NcAbaA and the neighboring ORF were maintained. ANID_00421 and NCU02611 shared a strong sequence similarity (E-value 0), which supported orthology between AbaA and NcAbaA based on microsynteny (Fig. 3.2).

Expression of *N. crassa asm-1* from the native promoter complements the *stuA1* mutant in *A. nidulans*

The loss of function mutant, *stuA1* produces shortened aerial stalks and conidia can be produced directly from either vesicles or metulae (Fig. 3.3 b and c) (Clutterbuck, 1969). I generated a Δs*tuA* mutant (Fig. 3.4) and it displayed a phenotype indistinguishable from that of *stuA1* (data not shown). In addition, I observed that conidia production in *stuA1* was less than wild type by ~190 fold (Fig. 3.5). *Neurospora*

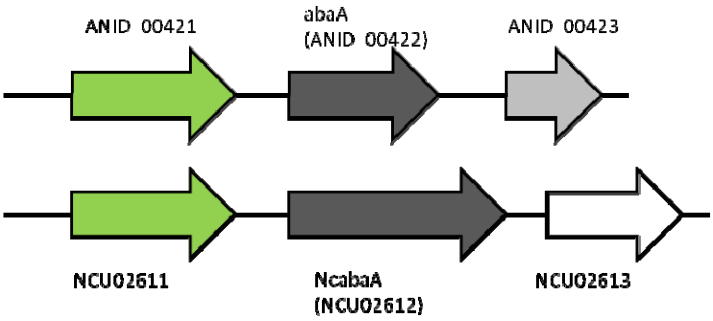

Figure 3.2 Microsynteny in *abaA* and *NcabaA* loci.
Amino acid sequences of the adjacent genes of *abaA* and *NcabaA* (ANID_00421and NCU02611, respectively) are highly conserved (E-value: 0), which suggests *abaA* and *NcabaA* share microsynteny. Although *abaA* and *NcabaA* have weak sequence similarity, existence of the microsynteny supports orthology between *abaA* and *NcabaA*.

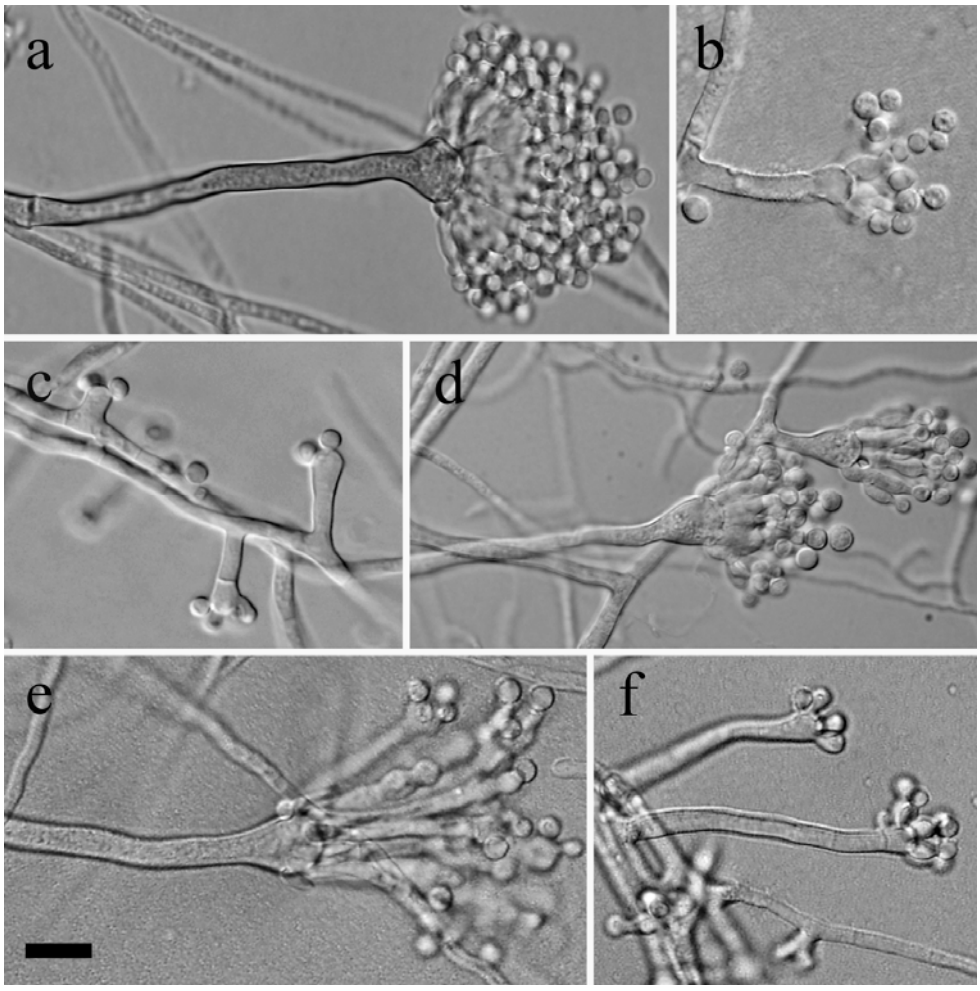

Figure 3.3 Complementation of defective conidiation in *stuA1* with expression of *asm-1* using the native promoter.
(a) A conidiophore of *A. nidulans* wild type (A773) displays two sterigmata: metulae and phialides, and conidia formation from the phialides. (b-c) Conidiophores of *stuA1* (Asus5). Aerial stalks are shortened, and conidia form directly from either metulae (b), or vesicles (c). (d) Conidiophores of TDC7.21 (*asm-1*(p)::*asm-1*; *stuA1*). Seventy percent of the conidiophores restored wild type conidiophore morphology, whereas 30% retained shortened aerial stalks. Sterigmata were normal in all of the conidiophores observed. (e-f) Conidiophores of TDC9.2 (*alcA*(p)::*asm-1*; *stuA1*). Over-expression of *asm-1* using *alcA* promoter caused abnormally differentiated sterigmata (e), and did not complement the shortened stalks in *stuA1*. Scale bar = 10 μm.

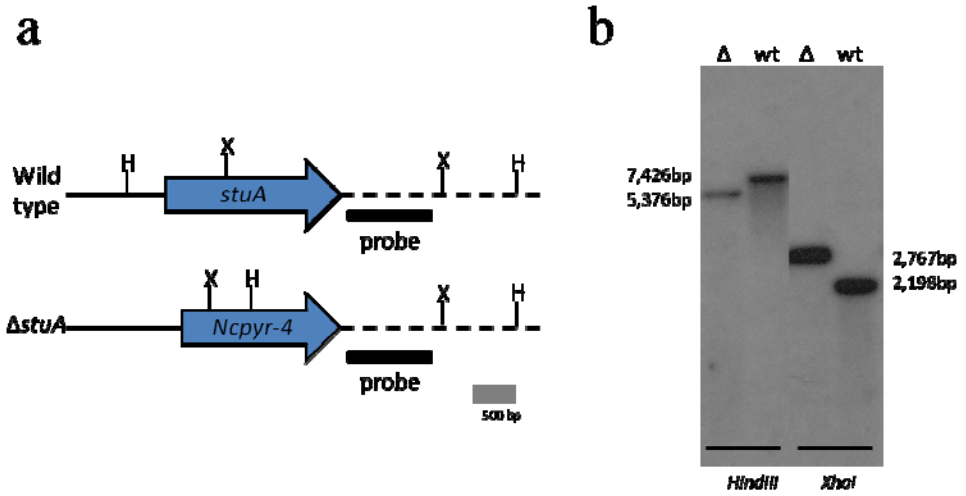

Figure 3.4 Verification of deletion of *stuA* in *A. nidulans*.
(a) A *stuA* knock-out construct. The top panel shows a *stuA* locus in *A. nidulans* wild type, and the bottom panel shows homologous replacement of *stuA* by *N. crassa pyr-4* (*Ncpyr4*) in Δ*stuA*. *HindIII* and *XhoI* restriction enzyme sites (as 'H' and 'X', respectively) were indicated. (b) Sothern blot analysis verified *stuA* was replaced by *N. crassa pyr-4*. Digested genomic DNA of Δ*stuA* (as 'Δ') and wild type (A4, as 'wt') by *HindIII* (5,376 bp for Δ*stuA* and 7,426 bp for wild type) and *XhoI* (2,767 bp for Δ*stuA* and 2,198 bp for wild type) exhibited the expected size.

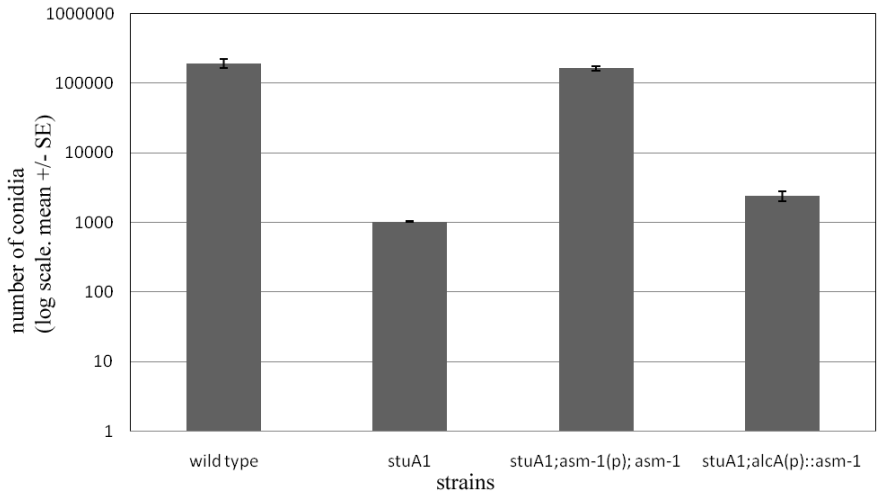

Figure 3.5 Complementation of defective conidia yield in *stuA1* by expression of *asm-1*. Conidia yield in *stuA1* is less than wild type by ~190 fold, and expression of *asm-1* using its native promoter (*asm-1*(p)::*asm-1*; *stuA1*) increases conidia yield by ~160 fold in *stuA1* (TDC7.21). In contrast, expression of *asm-1* using *alcA* promoter (*alcA*(p)::*asm-1*; *stuA1*) slightly increases conidia yield in *stuA1* by ~2.3 fold (TDC9.2). This experiment was performed in triplicate, and standard errors (SE) were indicated above the each bar.

crassa Δ*asm-1* exhibits slow mycelia growth, slow germination of conidia, and shortened aerial hyphae in conidiophores. Taken together, these data suggest that ASM-1 is involved in *N. crassa* development including macro-conidia formation (Aramayo et al., 1996). I expressed *N. crassa asm-1* using the native promoter in *stuA1* (TDC7.21). A single copy integration of *N. crassa asm-1* was verified by Southern blot analysis (Fig. 3.6). All of the observed conidiophores produced normal sterigmata, but 30% continued to exhibit shortened aerial stalks (Fig. 3.3d).

Expression of *asm-1* increased conidia yield by ~160 fold in *stuA1* to produce numbers similar to the number of conidia wild type produced (Fig. 3.5). This suggested that StuA and ASM-1 have a conserved biochemical function in conidiation.

In contrast, over-expression of *asm-1* using the *alcA* promoter (TDC9.2 and TDC9.5) did not complement defective conidiation in *stuA1*. Conidiophores produced in TDC9.2 and TDC9.5 retained the shortened aerial stalks (Fig. 3.3f), and the abnormal cell differentiation in sterigmata phenotype remained in many conidiophores (Fig. 3.3e). Conidia yield in *stuA1* was slightly increased by ~2.3 fold by over-expression of *asm-1*, but was still ~80 fold less than wild type (Fig. 3.5). These data suggested that the expression level and/or upstream sequence of *asm-1* were critical to determine the ability of ASM-1 to complement defective conidiation in *stuA1*.

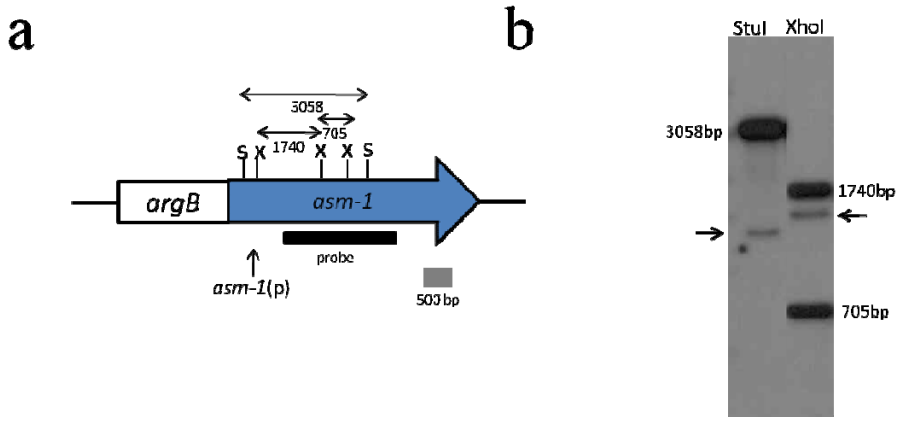

Figure 3.6 Southern blot analysis for a single copy integration of *N. crassa asm-1*.
(a) A construct to express *N. crassa asm-1* using the native promoter in *stuA1*. *StuI* and *XhoI* restriction enzyme sites (as 'S' and 'X', respectively), and the expected band sizes were indicated. (b) Southern blot analysis was performed to verify a single copy integration of the *N. crassa asm-1* construct in TDC7.21 (*asm-1*(p)::*asm-1*; *stuA1*). Digested genomic DNA of TDC7.21 by *StuI* and *XhoI* exhibited bands of the expected size and number. In addition to the band with the expected size, another extra band marked as an arrow indicates that a single copy of the construct is integrated ectopically in *stuA1*.

Expression of *N. crassa asm-1* does not complement defective sexual development in *stuA1*

StuA and Asm-1 are involved in both asexual and sexual development in *A. nidulans* and *N. crassa*. For example, *stuA1* and Δ*stuA* did not produce Hülle cells and cleistothecia, and Δ*asm-1* does not produce protoperithecia (Aramayo et al., 1996; Clutterbuck, 1969). Expression of *N. crassa asm-1* using either the native *N. crassa* promoter or the *alcA* promoter did not complement the defective sexual development *stuA1*. This suggested that StuA and ASM-1 might not have interchangeable biochemical functions in sexual development or that expression from the promoter constructs in this study was not sufficient for sexual development. A construct containing 1.7 kb upstream of the *stuA* upstream sequence complements defective conidiation but not sexual development in Δ*stuA* (Wu and Miller, 1997). To complement the sexual defects in Δ*stuA*, about 3.5 kb upstream of the *stuA* start codon is sufficient (Wu and Miller, 1997). Therefore, it is possible 1.5 kb upstream sequence of the *asm-1* start codon in our experiment was not sufficient to express *asm-1* for complementing sexual defects in *stuA1*.

Expression of *NcabaA* did not complement defective conidiation in *abaA14*

abaA14 is a temperature-sensitive mutant in *A. nidulans*. At 28ºC, conidiation occurs normally in *abaA14*, whereas at 37 and 42°C, *abaA14* produces ‘abacus’-like sterigmata with no conidia formation (Clutterbuck, 1969). Δ*NcabaA* (*N. crassa* knock-out project, NCU02611 null mutant) exhibits no detectable phenotypes in

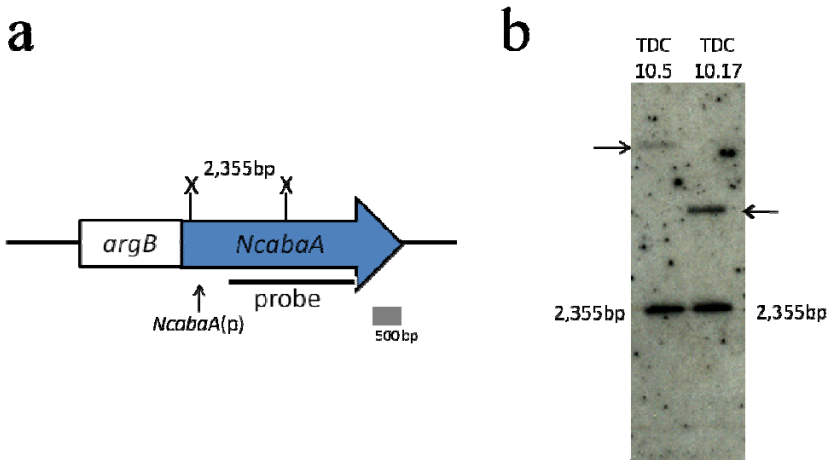

Figure 3.7 Southern blot analysis for a single copy integration of *NcabaA*.
(a) A construct to express *NcabaA* using its native promoter in abaA14. *XhoI* restriction enzyme sites (as 'X'), and the expected band sizes were indicated. (b) Southern blot analysis was performed to verify a single copy integration of the *NcabaA* construct in TDC10.5 and TDC10.17 (*NcabaA*(p)::*NcabaA*; *abaA14*). Digested genomic DNA of TDC10.5 and TDC10.17 by *XhoI* exhibited bands of the expected size and number. In addition to the band with the expected size, another extra band marked as an arrow indicates that a single copy of the construct is integrated ectopically in *abaA14*.

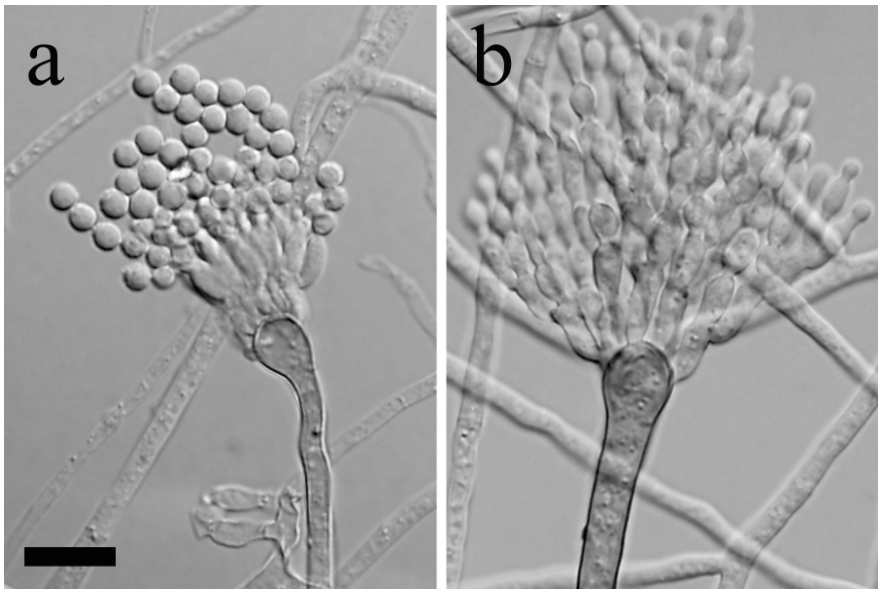

Figure 3.8 Expression of *NcabaA* does not complement defective conidiation in *abaA14*. (a) A conidiophore of *A. nidulans* wild type (A773) produces a vesicle and ordered sterigamta (a metulae → a phialide) and conidia. (b) A conidiophore of *aba14* produces abacus-like sterigmata with no conidia formation. Expression of *NcabaA* using either its native *N. crassa* promoter, *alcA* promoter, or *A. nidulans abaA* promoter displayed similar conidiophore morphology to *abaA14* (b) (data not shown). Scale bar = 10 μm.

macroconidiation compared to wild type *N. crassa*. In contrast, Δ*NcabaA* does not produce microconidia, which suggests that NcAbaA is involved in conidiation in *N. crassa*.

We expressed a single copy of *NcabaA* using its native promoter (TDC10.5 and TDC10.17) (Fig. 3.7) and examined the conidiation phenotypes at 28 and 42°C. Conidiophore morphology in TDC10.5 and TDC10.17 was normal at 28°C, whereas it was mutant-like at 42°C compared to wild type (Fig. 3.8), In contrast, expression of *A. nidulans abaA* using the native *A. nidulans* promoter complemented defective conidiation in *abaA14* (data not shown). Next I expressed *NcabaA* using both the *alcA* promoter (TDC11.1 and TDC11.3) and *A. nidulans abaA* 'chimeric' promoter (TDC12.1 and TDC12.2) in *abaA14*. Neither of these constructs, complemented defective conidiation in *abaA14* (Fig. 3.8). Taken together, these data suggest that AbaA and NcAbaA do not share conserved biochemical activity in conidiation.

StuA::GFP and AbaA::GFP localized to nuclei

Aspergillus nidulans stuA coding sequence contains an APSES DNA-binding motif (Dutton et al., 1997). I expressed StuA::GFP fusion proteins and found that they localized to nuclei, which was consistent with previous findings of StuA::GFP expressed by the constitutive *gpdA* promoter (Suelmann et al., 1997).

A. nidulans AbaA is an ATTS/TEA transcription factor (Andrianopoulos and Timberlake, 1994). I tagged AbaA by fusing GFP at the C-terminus by insertion into the native *abaA* locus (Fig. 3.9). AbaA::GFP localized to nuclei of phialides (Fig. 3.10). No

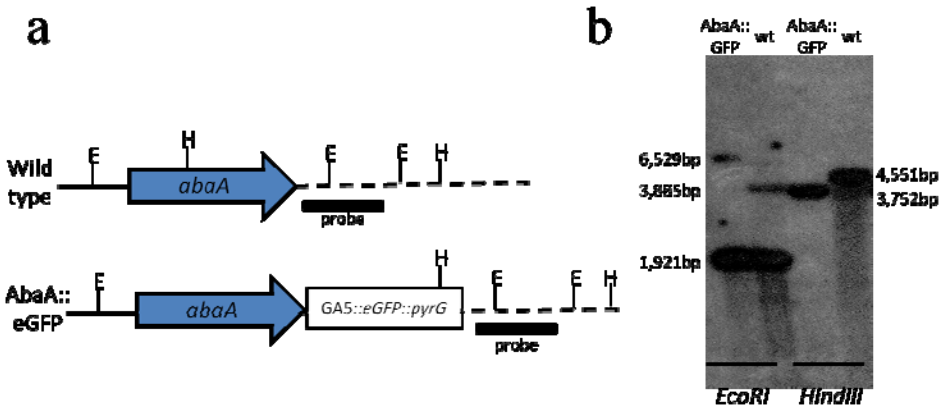

Figure 3.9 AbaA::eGFP fusion protein expressed by *abaA* native promoter.
(a) A AbaA::eGFP fusion construct. The top panel shows the *abaA* locus in *A. nidulans* wild type, and the bottom panel shows eGFP-tagged *A. nidulans abaA* at its native locus (AbaA::eGFP) (fused to pFN03 fragment including *GA5*::*eGFP*::*Aspergillus fumigatus pyrG* (*pyrG*)). *EcoRI* and *HindIII* (as 'E' and 'H', respectively) were indicated. (b) Southern blot analysis verified the eGFP construct was integrated at the native *abaA* locus. Digested genomic DNA of TDC8.1 (as 'AbaA::eGFP') and wild type (A4, as 'wt') by *EcoRI* (1,921 and 6,529 bp for AbaA::eGFP, and 1,921 and 3,885 bp for wild type) and *HindIII* (3,752 bp for AbaA::eGFP and 4,551 bp for wild type) exhibited the expected size.

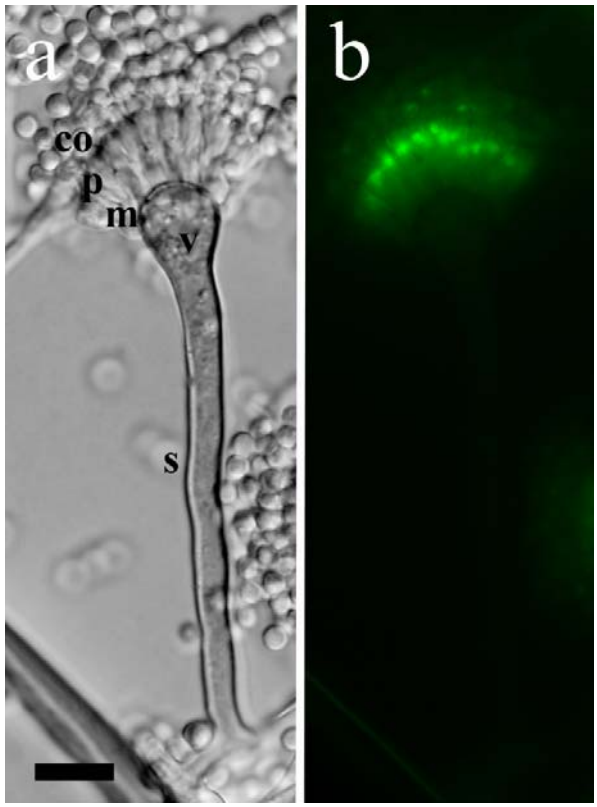

Figure 3.10 AbaA::eGFP localization.
Aspergillus nidulans AbaA::eGFP fusion protein was expressed at the native *abaA* locus, and the AbaA::eGFP localization was examined in different tissue types including germlings, vegetative hyphae, a stalk (‘s’), a vesicle (‘v’), metuale (‘m’), phialides (‘p’), and conidia (‘co’). (a-b) a conidiophore of TDC8.1 with a DIC image (a) corresponding to a fluorescence image (b). AbaA::eGFP localizes to nuclei, and the localization is spatially concentrated to phialides.

GFP signal was detected in germlings, vegetative hyphae, and other cell types of the conidiophore, which suggests that AbaA is a phialide-specific protein involved in regulating cell differentiation for conidiation.

DISCUSSION

AbaA and StuA are transcription factors (ATTS/TEA and APSES, respectively) involved in conidiation in *A. nidulans* (Andrianopoulos and Timberlake, 1994; Wu and Miller, 1997). *abaA14* forms abnormal conidiophores without producing conidia in a temperature dependent manner, and *stuA1* produces shortened aerial stalks and conidiophores with reduced conidia yield (Clutterbuck, 1969).

Orthologous or homologous StuA proteins have been characterized in a diverse group of fungi including *N. crassa*, *S. cerevisiae*, *C. albicans*, *F. oxysporum*, *P. marneffei*, and *U. maydis* (Aramayo and Metzenberg, 1996; Borneman et al., 2002; Garcia-Pedrajas et al.; Gimeno and Fink, 1994; Ohara and Tsuge, 2004; Stoldt et al., 1997) revealing conserved functions in cell differentiation including conidiation and pseudohyphal growth. Compared to StuA, orthologous AbaA proteins are readily identifiable in fewer fungal species, including *Aspergillus spp. Penicillium spp*. (Andrianopoulos and Timberlake, 1994; Borneman et al., 2000), *S. sclerotiorum* and *U. maydis*. To understand how these two transcription factors evolved to control conidiation in fungi, we assessed the conservation of function of AbaA and StuA in conidiation in two evolutionarily distant ascomycetes, *A. nidulans* and *N. crassa*.

ASM-1 is a previously characterized StuA ortholog from *N. crassa* (Aramayo et al., 1996), and expression of *N. crassa asm-1* using the native promoter complemented defective conidiation in *stuA1*. Taken together with the similar phenotypic impacts of knocking out *asm-1* in *N. crassa*, this suggests that the StuA family proteins have a conserved biochemical activity in conidiation in both *A. nidulans* and *N. crassa*. In contrast, over-expression of *asm-1* using the *alcA* promoter did not complement defective conidiation in *stuA1*. Thus, it seems that upstream promoter sequence is critical for proper gene expression to regulate development.

I suspect the importance of upstream promoter sequence may be complicated in the sexual development phenotype. Although expression of *asm-1* using its native promoter complements defective conidiation, the same expression construct does not complement sexual defects in *stuA1*. A similar phenotype was also observed in our previous study of *medA* and the *N. crassa* ortholog *acon-3*, where expression of *acon-3* using the native *N. crassa* promoter complemented defective conidiation but not sexual defects in *A. nidulans medA15* and Δ*medA* (Chapter II). These data imply that a more thorough analysis of the upstream promoter regions of these genes may reveal sequences important for regulation of sexual development. Previously, Wu and Miller showed that about 3.5 kb upstream of the *stuA* start codon complements both asexual (conidiation) and sexual defects in Δ*stuA* (Wu and Miller, 1997). Therefore, it is possible that expanded promoter sequence and/or the native *A. nidulans stuA* promoter would be necessary to express *N. crassa asm-1* to complement defective sexual cycle in *stuA1*.

Another explanation for why expression of *asm-1* did not complement sexual defects in *stuA1* might be that a function of ASM-1 in sexual cycle became specialized to *N. crassa* during evolution, and thus was not sufficient to rescue normal *A. nidulans* sexual development. Boreneman *et al*. showed that expression of *P. marneffei stuA* using its native promoter including either 1.6 kb or 3.6 kb upstream sequence complemented defective conidiation but not sexual cycle in *A. nidulans* Δ*stuA*. Given *P. marneffei* is an asexual species, it is possible *P. marneffei* lost a function for the sexual cycle, which is required for complementing the sexual defect in Δ*stuA* (Borneman et al., 2002). The relationship between promoter sequence and ability to complement sexual defects remains to be examined in future studies.

NcAbaA showed a very low sequence similarity to *A. nidulans* AbaA, but microsynteny with the neighboring gene strongly implies orthology. Deletion of *NcabaA* did not affect macro-conidiation in *N. crassa*. Thus, it was not surprising I have not seen complementation of defective conidiation in *A. nidulans abaA14* by expression of *NcabaA*. However, previous research on *flbD* and the *N. crassa flbD* ortholog, *rca-1*, demonstrates that expression of an orthologous gene with non-analogous function in conidiation can complement the biochemical activity for conidiation. For example, deletion of *rca-1* does not show detectable defects in macro-conidiation in *N. crassa*. However, expression of Rca-1 using the native *N. crassa* promoter complements defective conidiation in the *A. nidulans flbD* mutant (Shen et al., 1998). In this study the lack of complementation between AbaA and NcAbaA suggests such divergence in sequence has eliminated the potential for complementation of biochemical activity.

I demonstrated that AbaA::GFP fusion proteins expressed from the native *A. nidulans abaA* locus localized to nuclei, and the localization was detectable in phialides only. Transmission electron microscopy (TEM) images show that *abaA1* has a defect in phialides differentiation. For example, the abacus-like cells in *abaA1* do not have extra cell walls required for formation of inner cell walls in an initial conidium compared to wild type (Sewall et al., 1990a). These data suggest that AbaA proteins might function for conidiation specifically by regulating phialide differentiation. *A. nidulans* forms two uninucleate sterigmata, metulae and phialides. Conidia chains are generated from phialides basipetally (Adams et al., 1998). In contrast, proconidia chains develop acropetally without phialide cells for macro-conidiation in *N. crassa* (Springer and Yanofsky, 1989). The phialides-concentrated spatial expression of AbaA might be the potential determinant of its function in conidiation in *A. nidulans.* Thus, the morphological differences in conidiation between *A. nidulans* and *N. crassa* may explain the reason for the apparent divergence in function of NcAbaA and AbaA. It is noteworthy that Δ*NcabaA* does not produce microconidia (unpublished data). Unlike macro-conidia, micro-conidia are uninucleate and their formation is phialidic as in *A. nidulans* conidiation (Maheshwari, 1999). Therefore, lacking micro-conidiation in Δ*NcabaA* could be another example to support AbaA might have a function in conidiation to specific cell types.

Previously, I suggested three patterns for the evolution of genes that regulate conidiation in ascomycetes. These include: (i) non-homologous genes with analogous roles (the key regulators, *brlA* and *fl*), (ii) orthologs with complementary biochemical

function that lack an analogous role in conidiation (*flbD*, and *rca-1*), and (iii) orthologs with conserved biochemical function and an analogous role in conidiation (*medA* and *acon-3*) (Chung et al., 2011). To this list I can now add *stuA* and *asm-1* that also fall into pattern (iii). In addition, I can add a fourth pattern that includes *abaA* and *NcAbaA* data, which is (iv) orthologs with non-complementary biochemical function and with an analogous role in conidiation. Given that *A. nidulans* and *N. crassa* diverged from the ancestral species approximately 320 million years ago (Taylor and Ellison, 2010) and that the conidiophores bare striking morphological and ontogenic differences, it is likely that there are multiple patterns for evolution of conidiation regulators in ascomycetes. A degree of conservation of function in conidiation regulators could guide us to understand how these regulators evolve to properly control conidiation.

CHAPTER IV

CONCLUSION

DISTINCT EVOLUTIONARY PATTERNS IN CONIDIATION REGULATORS IN ASCOMYCETES

I hypothesized that a conidiation regulatory pathway was present in the ancestral species, and became specialized through divergent evolution to lead to the morphological and functional diversity in the extant species. Conversely, if my hypothesis is not supported by our data, it would suggest conidiation has arisen independently through convergent evolution. To understand the evolution of conidiation regulators in ascomycetes, I performed cross-species complementation with three *A. nidulans* conidiation regulatory genes*, abaA*, *stuA*, and *medA* and their *N. crassa* orthologs, *NcabaA*, *asm-1*, and *acon-3*. Expression of *asm-1* and *acon-3* using the native *N. crassa* promoters complemented defective conidiation in the *A. nidulans stuA* and *medA* mutants (Chapters II and III, respectively). In contrast, expression of *NcabaA* using either the native *N. crassa* promoter, *alcA* promoter, or the *A. nidulans abaA* promoter did not complement defective conidiation in the *A. nidulans abaA* mutant (Chapter III). These data suggest that the biochemical activity to regulate conidiation was conserved in the orthologous StuA (ASM-1) and MedA (ACON-3) protein, but not in the orthologous AbaA (NcAbaA) protein. In addition, these data suggest that the conidiation pathway did not simply diverge, and there are at least four distinct patterns of evolution in conidiation regulators in ascomycetes. In each model shown, the

orthologs illustrated with the same shape have an analogous role in conidiation, and the orthologs with the same color have a complementary biochemical function.

First, we suggest an evolutionary pattern of non-homologous genes with analogous roles in conidiation (Fig. 4.1). BrlA and Fl are the key regulators of conidiation in *A. nidulans* and *N. crassa*, which means that they have analogous roles in conidiation in each species. However, they are not homologous to each other, and there are no homologs of BrlA and Fl found in *N. crassa* and *A. nidulans*, respectively. Possible explanations for this pattern include, BrlA existed in the ancestral species to regulate conidiation and was retained in *A. nidulans* but lost in *N. crassa* during evolution. Similarly, Fl may have existed in the ancestor to regulate conidiation and was retained in *N. crassa* but lost in *A. nidulans*. Alternatively, BrlA or Fl did not have a function in conidiation in the ancestral species, but later evolved the ability to regulate conidiation through convergent evolution. With the data in this study, it is difficult to conclude how these key regulators evolved. Given that BrlA and Fl are found only in *Aspergillus* and *Neurospora* species and the closely related *Penicillium* species (within the same family Trichocomaceae) and *Podospora* species, respectively, both specific regulators evolved to control specialized conidiation machinery in these species.

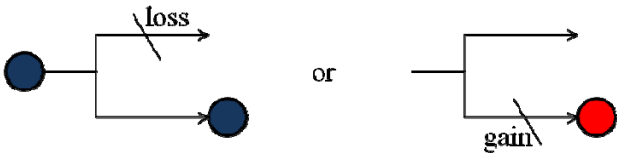

Figure 4.1 Pattern I: non-homologous genes with analogous roles in conidiation.

Second, we suggest an evolutionary pattern of orthologs with retained biochemical function that lack analogous roles in conidiation (Fig. 4.2). Complementation of the *A. nidulans flbD* mutant using the *N. crassa* ortholog (*rca-1*) reveals this pattern (Shen et al., 1998). Deletion of *rca-1* causes no detectable effect on macroconidiation in *N. crassa*, whereas disruption of *flbD* caused delayed conidiation in *A. nidulans*. Thus, these genes do not share an analogous role in conidiation in each species. However, expression of *rca-1* complements defective conidiation of the *A. nidulans flbD* mutant, which supported RCA-1 and FlbD sharing a complementary biochemical function. One explanation for this observation was that FlbD in the ancestral species had a function in conidiation, and its function was retained in *A. nidulans*, but lost in *N. crassa* through divergent evolution. Alternatively, FlbD did not have a function in conidiation in the ancestral species, but the ability of FlbD to regulate conidiation was obtained in *A. nidulans*. A survey of the function of FlbD orthologs in more species, especially species intermediate to *N. crassa* and *A. nidulans* might shed light on this question.

In related work from our project, not discussed in this dissertation, we found similar evolutionary patterns to FlbD and RCA-1 in the result of FluG and WetA. Disruption of either FluG or WetA causes abnormal conidiation in *A. nidulans*, whereas phenotypes associated with macroconidiation are not detectable in the orthologous *N. crassa fluG* (*NcfluG*) and *wetA* (*NcwetA*) deletion mutants. Expression of *NcfluG* and *NcwetA* using the native *N. crassa* promoter complemented defective conidiation in the

A. nidulans fluG and *wetA* mutant, respectively (Upadhyay et al., manuscript in preparation).

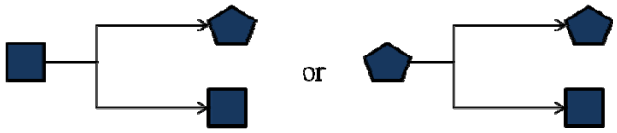

Figure 4.2 Pattern II: orthologs with retained biochemical function that lack analogous roles in conidiation.

Third, we suggest an evolutionary pattern of orthologs with conserved biochemical function and a role in conidiation in both organisms (Fig. 4.3). In this study, expression of *N. crassa acon-3* and *asm-1* using their native promoters complemented defective conidiation in the *A. nidulans medA* and *stuA* mutants. Both MedA and Acon-3, and StuA and Asm-1 have a role in conidiation in *A. nidulans* and *N. crassa*. The most parsimonious explanation of this pattern is that the function of these proteins in the ancestral species was regulation of conidiation and it was retained in the extant species.

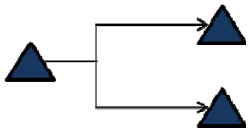

Figure 4.3 Pattern III: orthologs with conserved biochemical function and a role in conidiation in both organisms.

Fourth, we suggest an evolutionary pattern of orthologs with biochemical function not conserved and a role in conidiation (Fig. 4.4). In this study, expression of the *N. crassa abaA* ortholog (*NcabaA*) did not complement defective conidiation of the *A. nidulans abaA* mutant. Deletion of *NcabaA* did not lead to detectable defects in macroconidiation in *N. crassa*, however, it did result in a loss of microconidiation. Amino acid sequences of AbaA and NcAbaA share a low sequence similarity, which suggests that the sequence of these orthologs has diverged significantly since evolution from the last common ancestor. It is possible that the divergence that led to two species with basipetal macroconidiation only (*Aspergillus*) vs. acropetal macroconidiation and phialidic microconidiation influenced the divergence of AbaA. Thus, both are involved in asexual reproduction, but the loss of sequence similarity yielded loss of the ability to complement biochemical function.

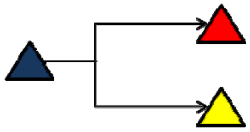

Figure 4. 4 Pattern IV: orthologs with biochemical function not conserved and a role in conidiation.

SUMMARY

Divergent evolution describes when related species from a common origin evolve distinct traits. Evolution of transcription factors (TFs) is a well-characterized example of divergent evolution. Most bilateral animals share core TFs that originated from a common ancestor, and the TFs exhibit similar functions in regulation of

embryonic development (Hsia and McGinnis, 2003). For example, Hox TFs determine embryonic regions on the head-tail axis in many vertebrates and invertebrates (Veraksa et al., 2000). In plants, MADS-box genes encode TFs to regulate floral architecture and flowering time, and more than 40 genes are known in *Arabidopsis*. Orthologs of these floral homeotic MADS-box genes are found in angiosperms and gymnosperms, which are estimated to have diverged 300 million years ago. Moreover, homologs of MADS-box genes are identified in distantly related, non-flowering plants including moss, and ferns (Ng and Yanofsky, 2001). These examples reflect similar findings to this work demonstrating that modified genes encoding TFs can lead to morphological divergence in plants and animals.

In contrast, convergent evolution describes the scenario where unrelated lineages acquire a similar biological trait in a similar environment. Examples of convergent evolution are found in unrelated aquatic vertebrates sharing a streamlined shape (Futuyma, 1998), and two distinct groups of mammals (cows and monkeys) with a fermentative foregut exhibiting similar physicochemical properties of stomach lysozymes (Stewart et al., 1987).

My hypothesis which supports divergent evolution of conidiation pathway are likely reasonable for Pattern II, III, and IV. That is, regulators comprising the pathway played a role in conidiation in the ancestral species, and evolved to have the role in conidiation either (i) conserved (*medA*, *stuA*, and *abaA*), or (ii) lost in the individual species (*fluG*, *flbD*, and *wetA*).

The most striking observation of conidiation regulators in *A. nidulans* and *N. crassa* is that the key regulators of conidiation in each species are not homologous, and orthologs of each regulator are found only in a lineage-specific manner (Pattern I). My hypothesis cannot explain this evolutionary pattern. Instead, through convergent evolution, BrlA or Fl could evolve as a key regulator for conidiation in each species, although they may have not had a function in conidiation in the ancestral species. Since both BrlA and Fl are necessary and sufficient to induce conidiation in each species, gaining the functional analogy as the key regulator could be advantageous for both species to reproduce and survive under a wide range of environments.

Conidiation of *A. nidulans* and *N. crassa* involves formation of morphologically different conidiophores and conidia. We suggest that both divergent and convergent evolution led to construction of the extant conidiation regulatory pathways in ascomycetes. In addition, I speculate that different types of evolution applied to the ancestral genes are the critical determinants of distinct morphological features in conidiophores and conidia specialized to each extant species.

FUTURE WORK

I have addressed my hypothesis by assessing conserved function of conidiation regulators between *A. nidulans* and *N. crassa*. I have characterized four evolutionary patterns of conidiation regulators in ascomycetes. However, to understand evolution of conidiation machinery more clearly, several questions remain to be elucidated.

(i) Would expression of the *A. nidulans* ortholog complement defective macroconidiation in the corresponding *N. crassa* mutant? For example, would expression of *medA* and *stuA* complement defective macroconidiation in the *N. crassa acon-3* and *asm-1* deletion mutant? Addressing this question will confirm the conserved biochemical function of the conidiation regulators between *A. nidulans* and *N. crassa*. If the result of this study is not consistent with the result presented in this study, that would raise additional questions including the importance of proper expression of the ortholog in a species-specific manner (both timely and spatially). The issue could be further studied in (ii).

(ii) Would different upstream sequences affect the ability of the *N. crassa* orthologs to complement defective asexual and sexual development in the *A. nidulans* mutants? Expression of *N. crassa* ortholog (*acon-3* and *asm-1*) complemented defects in conidiation but not in sexual development of the *A. nidulans medA* (Chapter II) and *stuA* (Chapter III) mutant. In addition, expression of *asm-1* using its native promoter complemented defective conidiation in the *A. nidulans stuA* mutant, but expression of *asm-1* using the *alcA* promoter did not. These data suggest that upstream sequence of the ortholog is critical to determine the ability of complementation. Therefore, expression of the *N. crassa* ortholog using either an expanded promoter sequence or *A. nidulans* promoter would address this question. Related to (i), this future work will increase our understanding of the importance of upstream sequences, and possibly an effect of the differentially evolved upstream sequences on conserved biochemical activity of the gene product in conidiation.

(iii) How do the conidiation regulators coordinate conidiation in each species? The data obtained in this study focused largely on the activities of each regulator individually. Future work should examine the interactions between these regulators. For example, localization of MedA::GFP (Chapter II) and AbaA::GFP (Chapter III) expressed by the native promoter could be studied in the *A. nidulans* mutants compared to in wild type. If the MedA::GFP or AbaA:;GFP is mislocalized in a specific mutant, it would suggest that the function of the specific gene links to normal function of MedA and AbaA. Additionally, other regulators could be tagged using GFP at the native locus and localization of the fusion proteins could be observed in wild type, other conidiation mutants, and the complemented strains.

(iv) How are some of the conidiation regulators in *A. nidulans* and *N. crassa* able to show similar biochemical function in conidiation although these two species do not share the conserved key regulator? Function of several conidiation regulators in *A. nidulans* are directly or indirectly associated with the key regulator BrlA. For example, *abaA* expression is activated by *brlA*, and normal temporal and spatial expression of *brlA* is impaired in the *medA* and *stuA* mutant. There could be unknown regulators interacting with the key regulators BrlA and Fl to control conidiation in each species. If these unknown factors are conserved in *A. nidulans* and *N. crassa*, they might be able to cooperate with orthologs to complement the defective conidiation of the mutant without existence of the key regulators conserved. To address this question, I could identify interacting factors to BrlA and Fl in each species. For example, identification of unknown peptides via affinity purification has been well-established in *A. nidulans* (Liu

et al., 2009). Using this technique, BrlA and Fl could be fused to S-tag for purification (Kim and Raines, 1993), and the purified protein complex could be characterized using mass spectrometry.

(v) How did the lineage-specific conidiation regulators evolve? For example, orthologs of *brlA* are found only in *Aspergillus* and *Penicillium spp*. (within the same family Trichocomaceae), and orthologs of *fl* are found only in *Podospora anserina* (within the same order Sordariales with *N. crassa*). Therefore, I speculate that *brlA* and *fl* evolved as specialized conidiation regulators in each lineage. Moreover, individual species in the closely related groups have distinct modes of conidiation. For example, a conidiophore of *A. fumigatus* and most of *Penicillium spp*. does not form metulae and swollen vesicles, respectively. In addition, *P. anserina* does not develop macroconidia (but microconidia are produced) for which *N. crassa fl* serves as a key regulator. In this study, I elucidated a larger scale of evolutionary patterns of conidiation regulators. In order to understand in which evolutionary step these regulators either gained or lost their functions in conidiation, analysis of species at a closer evolutionary scale will be required.

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